

NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES

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OFFICE OF THE DIRECTOR

During 1985, the National Institute of Environmental Health Sciences completed its eighteenth year since its establishment. It continues as the only National Institutes of Health Institute headquartered away from Bethesda, Maryland.

Established in 1966, the Institute has developed from a small group occupying temporary laboratory facilities to one of the major toxicology research organizations in the world, with a staff of almost 1000; about 200 of whom are doctoral level scientists. At the same time, it has developed a broad array of grant and contract mechanisms which permit the support of studies and investigations in non-Federal laboratories within both universities and the private sector.

Further, since its inception the Institute has been assigned a variety of new tasks, paramount among which is managerial responsibility for the National Toxicology Program. In addition, the Institute has been designated a World Health Organization participating Institute in the International Program on Chemical Safety and has been assigned major responsibility for a variety of international agreements.

The Institute journal, Environmental Health Perspectives, first published in 1972, has become a major publisher of topical conferences on new and emerging environmental health science problems, and has attracted the attention of scientists throughout the world.

In summary, the Institute, established in response to emerging concern for the public health impacts of the environment, has played a major role in elucidating the human health impacts resulting from our efforts to control and modify our environment.

This summary provides a broad overview of the following more detailed reports of the Institute's scientist leaders and the research conducted in their laboratories or which the Institute supports by grant or contract.

Research Developments

In an effort to develop the data needed to anticipate the future health needs of survivors recovering from the effects of widespread exposure to methyl isocyanate (MIC) at Bhopal, India, the Institute--through its Toxicology Research and Testing Program--exposed a population of laboratory rats and mice to one-time and multiple exposures of MIC. These animals are being followed to determine what health effects may be expected to persist or to develop through the use of an animal model to obtain a better understanding of MIC toxicity.

Preliminary studies by investigators in the Biometry and Risk Assessment Program on the health risks posed to non-smokers by smokers who live in the same household showed data that suggest overall cancer risk rose steadily and significantly for non-smokers with each additional household member who smoked over an individual's lifetime. A second study suggested that women

who smoke experience delays in attaining a desired pregnancy, and are 3.4 times more likely to have taken more than a year to conceive compared to nonsmokers.

At the Harvard School of Public Health, a short-term in vivo animal test has been developed which predicts whether chemicals can cause emphysema, fibrosis, or chronic bronchitis. Studies using the system have shown that volcanic ash is relatively non-toxic. But the system showed that the combustion byproduct of automobile waste oil--proposed for use in kerosene-type home heaters--is highly toxic.

A new technology the Institute is using to study toxic chemicals is nuclear magnetic resonance spectroscopy. In the Intramural Research and Toxicology Research and Testing Programs, and in cooperation with Duke University, high magnetic field spectroscopy [magnetic resonance imaging (MRI)] has been employed in animals through the use of surface coils to trace metabolic changes in phospho-organic compounds which reflect toxic chemical damage to the liver. In these non-invasive studies using live animals, the Institute generates MRI images based on the hydrogen resonances in water which, in some cases, can distinguish tumor tissue from fat infiltrations in the liver. The application of MRI spectroscopy and imaging to animal toxicity studies is just beginning, but a rapid development of this technology is expected.

The Toxicology Research and Testing Program has stimulated the development of microencapsulation as a means for encasing otherwise volatile chemicals in a protective edible casing. This process keeps chemicals stable and allows exposures to be administered in animal feeding studies without the labor intensive aspects and the risk factor of gavage administration. Microencapsulation studies with trichloroethylene showed that toxic response with microencapsulated feeding was comparable to the response in animals exposed to the chemical by gavage.

The Lawrence Berkeley Laboratory has assembled a large computerized database which includes the results of long-term animal cancer studies conducted by the National Cancer Institute and the NTP through July 1980 as well as related long-term chronic experiments reported in scientific literature. Data and information from approximately 3,000 single sex, single species experiments involving 770 different compounds are contained in the database. Included is information on dose levels, length of exposure, histopathology, tumor incidence, a carcinogenicity potency index and its related statistical significance and the published author's judgment as to carcinogenicity of the compound. The data are presented in a format that allows comparisons among chemicals, species, target organs and other variables of interest. Putting all this information into a single database facilitates the use of this large body of information by scientists from a variety of disciplines. The database was published in the NIEHS journal, Environmental Health Perspectives, volume 58.

Staff Changes

During the year, the Institute named Dr. Martin B. Rodbell Scientific Director of its Intramural Research Program. He is a distinguished scien-

tist with many years of experience both as a senior investigator and as a science administrator within the NIH. Dr. Robell is an internationally recognized scientist on lipoprotein structure and metabolism; fat transport and mechanisms of blood triglyceride uptake by liver and adipose tissues and isolated fat cells; and the mode of action of hormones at the cellular and molecular level. Dr. Rodbell's predecessor as Scientific Director was Dr. Charles E. Carter.

This year, Dr. Wilford Nusser, Associate Director, Extramural Program, retired after 25 years of Government service including eight years with the Institute. The Institute is in the process of identifying a successor to Dr. Nusser.

Following a long illness, Dr. Hans Falk died last winter. Among the first group of senior scientists appointed to the staff of the Institute, Dr. Falk played a key role in the development of the Institute's Intramural Program and later in its health hazard assessment activities. With an international reputation for his work in carcinogenesis, Dr. Falk was also respected for his encyclopedic knowledge of the field and of the toxicity of chemicals. Beyond his accomplishments and standing within the scientific community, Dr. Falk was mentor to a generation of scientists and co-workers both within the Institute and elsewhere. An individual of critical scientific judgment, Dr. Falk was also known to his friends and coworkers as a gentle, sensitive, and caring person. The Institute was favored in having him on its staff.

Meetings and Publications

The Report of the Third Task Force for Research Planning in Environmental Health Sciences has been submitted to the House Appropriations Committee. Publication and public distribution of the Report is planned for early 1986. The report describes the rapid expansion of technology, the use of which will help provide knowledge about effects of chemicals at low doses, such as in instances of human exposure or in laboratory animals, or in tissue or microbe cultures. In addition the Report recommends directions for future research. The Institute staff, the National Advisory Environmental Health Sciences Council, and other review groups are currently studying the Report which is expected to provide the basis for Institute planning for several years.

This year also saw publication of the Report of the Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation of the National Toxicology Program Board of Scientific Counselors. The Report reviews the design and achievements of research conducted under the aegis of the NTP. The Panel found that the on-going NTP effort has benefited from the existing peer review system and is evolving scientifically in appropriate ways. With respect to quality assurance, the Panel found that NTP managers had in a number of instances anticipated Panel recommendations and had already implemented changes where necessary.

The Institute continued to serve as a focal point for international conferences on environmental health-related topics. Over the year these conferences included, "DNA Adducts: Dosimeters to Monitor Human Exposure to

Environmental Mutagens and Carcinogens," which was held in September 1984; "Conference on Health Effects of Acid Precipitation," held in November 1984; and the "Second Symposium on Estrogens in the Environment: Influences on Development," in April 1985. Papers from each of these meetings as well as other conferences held at the Institute will be compiled for publication in scientific journals including the NIEHS journal Environmental Health Perspectives, or in some cases as full length books, to serve as references for future research in these areas of inquiry.

GENETICS

OFFICE OF THE ASSOCIATE DIRECTOR FOR GENETICS Summary Statement

During FY 1985 the Office of the Associate Director for Genetics (OADG) continued to fulfill its role in the Genetic Toxicology programs of the Institute by serving as an expert consultant to the Director and the intramural research staff and by developing programs in the areas of genetics and environmental mutagenesis. The OADG has provided a focal point as well as planning and coordination functions in a number of areas of genetic toxicology including (1) international programs, (2) national programs, (3) committees, (4) collaborative studies and (5) collaborative research programs.

International Programs

US-Japan

The Associate Director for Genetics (ADG) is Chairman of the U.S. Panel on Environmental Mutagenesis and Carcinogenesis in the U.S.-Japan Cooperative Medical Science Program. The 3rd Joint Workshop of the Panel was held in Honolulu, Hawaii, December 19, 1984, on the topic of "Research Needs for Human Health Hazard Assessment in Food Mutagen Research." The Workshop was held in conjunction with the Symposium on Formation of Mutagens During Cooking and Heat Processing of Foods. A Joint Conference of the Panel was held in Shimoda, Japan, January 8-10, 1985, on the topic of "Mutagenesis and Carcinogenesis by Nitropyrenes and Cancer Chemotherapeutics."

ICPEMC

The Associate Director for Genetics attended the commission meeting of the International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC) held in Trois Ilets, Martinique, October 7-11, 1984, to review the work of the various Task Groups and Committees as well as to identify future projects and activities. The ADG also participated in a meeting of Committee 1 held in Research Triangle Park, North Carolina, January 23-25, 1985, to complete the report on evaluation of short-term tests for mutagenicity.

5th International Conference on Environmental Mutagens

As chairman of the organizing committee, the ADG presided over a meeting in Cleveland, Ohio, April 28-29, 1985, to choose the site of the conference which is to be held in 1989 and to draw up preliminary plans for the meeting.

National Programs

EPA Gene-Tox Program

The ADG has participated in several periodically-held meetings of the Coordinating Committee during the second phase of the program. The purpose of this phase is for the various Assessment Panels to evaluate the utility of the various test systems, to cross-index the data and to make recommendations for

appropriate batteries of tests for mass screening. The Coordinating Committee reviews the reports of the Panels and reviews the feasibility of panel activities in terms of the computerized data base.

NIEHS-Sponsored Workshops

The ADG organized a workshop on "Gene Cloning in Lower Eukaryotes" held at NIEHS, Research Triangle Park, North Carolina, November 30-December 1, 1984 to examine the utilization of the great number of mutant stocks of Neurospora crassa for understanding the causes and mechanisms of mutation.

The OADG coordinated NIEHS co-sponsorship of the "Symposium on Aneuploidy: Etiology and Mechanisms" held in Washington, D. C., March 25-29, 1985. The ADG was a member of the organizing committee. Co-sponsor was the U. S. Environmental Protection Agency.

The Proceedings of the NIEHS Workshop, "Genetic Consequences of Nucleotide Pool Imbalance," were published in April 1985. The book is edited by the ADG. Technical editing was done by Dr. William Sheridan, OADG.

The Proceedings of the NIEHS Workshop "DNA Adducts: Dosimeters to Monitor Human Exposure to Environmental Mutagens and Carcinogens" were published in Volume 62 of Environmental Health Perspectives. Guest editors are the ADG, Dr. Barton L. Gledhill, Lawrence Livermore National Laboratory, and Dr. William Sheridan, OADG.

Seminars

The ADG, in collaboration with Intramural Research Program staff, has developed a seminar series entitled "New Frontiers in Genetics" for the purpose of inviting scientists whose research is the vanguard of advances in their fields of genetics to present their work to the NIEHS scientific staff. Lectures dealing with fragile sites in human chromosomes, homeotic genes in *Drosophila*, and monoclonal antibodies to detect DNA-Adducts have been given by such eminent scientists as Michelle Le Beau, William McGinnis and Manfred Rajewsky.

Committees

Subcommittee on Environmental Mutagenesis

The ADG continues to chair the DHHS-CCERP Subcommittee on Environmental Mutagenesis. Topics covered included: Genetic Toxicology of Nitropyrenes, Reproductive Failure: Effects on Germ Cells, Reproductive Failure: Effects on the Embryo and Fetus, Sensitivity of Heterozygotes in Repair-Deficiency Syndromes: Ataxia-Telangiectasia, Genetic Toxicology of Non-Carcinogens, and Evaluation of Rodent Bioassays for Mutagenicity. These and other current issues of importance to government agencies concerned with genetic toxicology will continue to be addressed by the Subcommittee.

Collaborative Studies

WHO-International Program for Chemical Safety

The ADG is chairman of a working group of the International Program for Chemical Safety (IPCS) sponsored by the World Health Organization, the United Nations Environmental Program and International Labor Organization. A meeting of the coordinators for the evaluation of short-term in vivo tests for carcinogenicity was held in Brussels, Belgium, November 12-17, 1984, to present status reports of the ongoing investigations and to plan for the meeting of investigators in 1985. In conjunction with this meeting, a larger meeting which included experts from many countries was held to discuss proposed Plant Systems Collaborative Studies.

A meeting of the investigators participating in the in vivo collaborative study was held at Cap d'Agde, France, May 13-24, 1985, to review the data from the experiments and to draft workgroup reports. The overview reports, as well as approximately 90 investigator's reports, are being prepared in a form for later publication.

In April 1985 "Evaluation of Short-Term Tests for Carcinogens: Report of the International Programme on Chemical Safety's Collaborative Study on in vitro Assays" was published by Elsevier Science Publishers. The volume includes background and summary reports, and the individual investigator's reports. The ADG is one of the editors.

Collaborative Research Programs

Illinois State University

The data generated during the period that the contract was in force are being utilized as the basis for scientific reports which continue to be prepared for publication.

Public Lectures

F. J. de Serres

1. University of North Carolina, Chapel Hill, N. C., October 1, 1984, "Mutation-Induction at Specific Loci in a Eukaryotic Organism and Genetic Characterization of the Mutants."
2. 16th Annual Meeting of Environmental Mutagen Society, Las Vegas, Nevada, February 25-March 1, 1985, "National and International Programs in Environmental Mutagenesis and Carcinogenesis: The Satellite Symposium on Food Mutagens in Copenhagen."
3. 16th Annual Meeting of Environmental Mutagen Society, Las Vegas, Nevada, February 25-March 1, 1985, "Effect of the Heterokaryotic State of the uvs-2 allele in Neurospora crassa on Nitrous Acid-Induced Killings and Mutation."

4. Symposium on Aneuploidy: Etiology and Mechanisms, Washington, D. C., March 25-29, 1985, "Mechanisms to Stimulate Research on Assay Systems to Detect Aneuploidy."
5. North Carolina Society of Toxicology, Raleigh, N. C., April 24, 1985, "Do We Have Sound Approaches To Evaluate The Toxicity of Complex Environmental Mixtures?"
6. 4th International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-28, 1985, "Genetic Characterization of Mutagenic Activity of Environmental Mutagens in *Neurospora*."
7. 54th Annual Meeting of Genetics Society of America, Boston, MA, August 11-15, 1985, "Genetic Characterization of Mutagenic Activity in Environmental Chemicals at Specific Loci in Two-Component Heterokaryons of *Neurospora crassa*."

INTERNATIONAL PROGRAMS

INTERNATIONAL PROGRAMS
OFFICE OF THE ASSISTANT TO THE DIRECTOR FOR INTERNATIONAL PROGRAMS
Summary Statement

The Assistant to the Director for International Programs is responsible for the following areas:

Cooperation with the World Health Organization (WHO)

NIEHS has been designated by WHO as a Collaborating Center for Environmental Health Effects since 1975. As a Collaborating Center, NIEHS provides advice and scientific expertise to WHO headquarters and WHO Regional Offices, and assists them in the formulation of research programs related to the biomedical aspects of environmental pollution.

In 1979, WHO established the International Programme on Chemical Safety (IPCS), a cooperative undertaking involving WHO, the United Nations Environmental Programme, the International Labor Organization, and their member states. In October 1980, a cooperative agreement was signed between NIEHS and WHO, and NIEHS assumed the function of a Participating Institution (PI) within the IPCS for such activities as international evaluation of the biological effects of chemicals and health hazard assessments, and review and/or validation of methods for testing of mutagenicity, carcinogenicity, neurobehavioral toxicity, and toxicity to reproductive function. In September 1983 the Agreement was extended for another three years. A WHO Interregional Research Unit (IRRU), housed at NIEHS was established in 1981 to assist the Central Unit established at WHO headquarters in coordinating the activities of the IPCS/PI's. Since the inception of the Programme, numerous scientific experts from NIEHS have participated on IPCS committees, special consultations, conferences, and technical working groups. For example, during FY 1985, the Director, NIEHS, and the Assistant to the Director for International Programs, NIEHS, participated in the Fourth Meeting of the IPCS Programme Advisory Committee. This Committee, composed of 20 members from IPCS/PI's (including the Director, NIEHS) is the general oversight body providing advice on the policies and priorities of the IPCS. Also, in FY 1985, the Associate Director for Genetics, NIEHS, chaired a meeting of investigators participating in the second phase of the IPCS "Collaborative Study on Short-term Tests for Genotoxicity and Carcinogenicity". The purpose of this study is to evaluate short-term in vivo assays for mutagenicity and carcinogenicity. In addition, during FY 1985, NIEHS staff continued to review IPCS environmental health criteria documents, working papers, and proposed projects.

The objectives of the IPCS are: (1) to encourage international cooperation in the evaluation of the effects of chemicals on human health and on the quality of the environment; (2) to coordinate chemical testing and toxicological research to eliminate unnecessary duplication of efforts; (3) to develop international protocols for laboratory testing, epidemiological studies, and risk assessment; (4) to develop international guidelines and exposure limits for chemicals in air, water, and food and limits for hazardous chemicals in workplaces; (5) to develop response mechanisms for coping with chemical emergencies which may be international in scope; and (6) to promote training and development of manpower in areas and specialties necessary for the achievement of program goals. The IPCS provides an effective multilateral mechanism for dealing with global environmental health issues and avoiding costly duplication of national efforts to test and assess chemicals.

During FY 1985, NIEHS staff also participated in the Fifth International Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC) dealing with methods for the assessment of exposure to chemicals of both human and non-human biota. SGOMSEC is an IPCS activity sponsored jointly with the Scientific Committee on Problems of the Environment of the International Council of Scientific Unions.

NIEHS also collaborates with the WHO International Agency for Research on Cancer (IARC). Collaborative efforts include the establishment of a registry of workers exposed to particular pesticides which contain dioxin contaminants. During FY 1985, scientists from NIEHS and the U.S. National Toxicology Program (NTP) participated in a number of IARC sponsored expert working groups to prepare "IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans." These monographs collect all available relevant experimental and epidemiological data about a chemical or groups of chemicals to which humans are known to be exposed, and summarize the evidence for the carcinogenicity of chemicals and other relevant information.

US-China (Mainland) Cooperation

Cooperation between the United States and the People's Republic of China in the area of environmental health was initiated during 1980 under the US-PRC Agreement for Cooperation in the Science and Technology of Medicine and Public Health. NIEHS is a participant in the topic on public health and health services research, which includes concerns relating to environmental and occupational health. Exploratory discussions between both sides have been held during exchange visits with initial discussions centering around cooperation in the following areas: reproductive and developmental toxicology; validation of short-term test methods to detect and assess carcinogens, mutagens, and teratogens in the environment; and the extrapolation of laboratory animal data to man. NIEHS scientists have given research seminars at various institutions in the People's Republic of China, and have hosted the visits of numerous Chinese scientists to discuss research programs of mutual interest.

US-China (Taiwan) Cooperation

Collaborative studies between U.S. and Taiwanese scientists are carried out through a Cooperative Program in the Biomedical Sciences between the American Institute in Taiwan and the Coordination Council for North American Affairs. For the past three years, NIEHS scientists from the Biometry and Risk Assessment Program have collaborated with Taiwanese scientists in studies investigating the effects of accidental human exposure to polychlorinated biphenyls (PCBs) in Taiwan. Studies on the effects of PCB exposure on enzymatic activity in human tissues suggest a potential for substantial and persistent effects of these pollutants on human metabolism. During FY 1985, a team of experts, led by NIEHS scientists, visited Taiwan to initiate a collaborative field study aimed at conducting comprehensive medical evaluations of Taiwanese children exposed in utero to PCBs and related chemicals.

US-Finland Cooperation

A Memorandum of Understanding on Collaboration between NIEHS and the Finnish Institute of Occupational Health, Helsinki, was formalized in November 1982. Areas of mutual interest include the following: pharmacokinetics; reproductive

toxicology; neurobehavioral toxicology; genetic toxicology; epidemiology and risk assessment; and strategies for toxicological research priority settings. NIEHS and the Finnish Institute of Occupational Health cosponsored an "International Workshop on Occupational Hazards Caused by Polychlorinated Biphenyls and Chlorobenzenes in Capacitors and Transformers," in 1983 in Finland. The proceedings of this workshop were published in the NIEHS journal, Environmental Health Perspectives, Volume 60, May 1985.

US-India Cooperation

NIEHS and NTP scientists are cooperating with India by conducting experimental animal studies on the toxicity of methyl isocyanate, the chemical responsible for the world's worst industrial disaster which occurred in Bhopal, India in December 1984. During FY 1985, NIEHS also co-sponsored an international symposium on "After Bhopal - Implications for Developed and Developing Nations", and hosted the visits of several Indian scientists to discuss the toxicity of methyl isocyanate.

US-Italy Cooperation

Cooperation between American and Italian health scientists has taken place since 1977 under the Memorandum of Understanding between the U.S. Department of Health and Human Services and the Italian Ministry of Health. Activities under this agreement have focused mainly on efforts to understand the mechanisms of toxicity associated with exposure to 2,3,7,8 tetrachlorodibenzodioxin (TCDD). Of special interest is the accidental exposure of humans to dioxins resulting from the explosion of a chemical reactor in Seveso, Italy, in 1976. Since then, exchange visits have taken place to discuss potential future collaboration in the following areas: (1) Studies on the bioavailability of TCDD to organisms after the contaminant has been in soil for some period of time. The bioavailability of TCDD may well be a function of the type of soil, and experimental data are necessary in order to make better scientific risk assessments concerning the effects of accidental exposure to TCDD in the U.S. and Italian populations. (2) Studies on the chemical contamination of drinking water.

US-Japan Cooperation

Cooperation between American and Japanese scientists on environmental health problems takes place under two formal agreements: The US-Japan Cooperative Medical Sciences Program and the Agreement on US-Japan Cooperation in Research and Development in Science and Technology. Under the US-Japan Cooperative Medical Sciences Program, American environmental health scientists participate in the Panel on Environmental Mutagenesis and Carcinogenesis chaired by the Associate Director for Genetics, NIEHS. Joint areas of research focus on the detection of mutagenic and carcinogenic chemicals using both in vitro and in vivo test systems, and on monitoring human populations for evidence of exposure to mutagenic and carcinogenic chemicals. The Director, NIEHS, serves as a member of the Joint Committee which oversees the activities of the US-Japan Cooperative Medical Sciences Program. In July 1985, the Director, the Associate Director for Genetics, and the Assistant to the Director for International Programs, NIEHS, participated in the 21st US-Japan Joint Committee Meeting to review panel activities and discuss proposed future programs. Also, during FY

1985 the US-Japan Environmental Panel held a workshop on "Mutagenesis and Carcinogenesis by Nitropyrenes and Cancer Chemotherapeutics" and on "Research Needs for Human Health Hazard Assessment in Food Mutagen Research."

Under the US-Japan Agreement on Cooperation in Research and Development in Science and Technology, NIEHS participates in the toxicology program area in the counterpart working group on health. Cooperative activities under this agreement focus on the testing of chemicals for mutagenic, carcinogenic, and other toxic effects. In FY 1985, annual exchange visits between Japanese and NIEHS scientists continued, and both sides have shared information on test method development, which chemicals will be tested, and test results. Several test systems are being reviewed and validated in both U.S. and Japanese laboratories.

US-Sweden Cooperation

During FY 1985, the Swedish Work Environment Fund approached NIEHS to initiate collaborative activities in toxicology and environmental health. Particular areas of interest include genetic toxicology, neurotoxicology, and immunotoxicology. Preliminary discussions have been held to plan joint US-Sweden workshops in these areas.

US-USSR Cooperation

Collaboration between Soviet and American environmental health scientists is carried out under the auspices of two cooperative agreements between the United States and the Soviet Union. Under the Medical Science and Public Health Cooperative Agreement, scientists from both countries are conducting joint research on the effects of physical and chemical environmental agents on human health. 1985 was the thirteenth year of formal collaboration in environmental health research between the U.S. and U.S.S.R. Cooperative research efforts have involved formal workshops and exchange visits between scientists of both countries. The fifth workshop on the "Biological Effects of Physical Factors in the Environment" was held in the Soviet Union in FY 1985 to discuss the results of joint US-USSR studies to date. One of the projects is a duplicate experiment aimed at evaluating sensitive tests for determining the biological effects of electromagnetic fields on the nervous system and to validate research results obtained in the U.S. and the Soviet Union. Three Soviet scientists visited NIEHS laboratories in FY 1985 to work on the duplicate experiment.

NIEHS also participates in the US-USSR Agreement on Cooperation in the Field of Environmental Protection which is administered for the United States by the Environmental Protection Agency. The Director, NIEHS, serves as DHHS representative to the Environmental Protection Agreement and co-chairman of the working group concerned with the biological and genetic effect of pollution. Exchange visits under this Agreement have been conducted in research areas concerned with the mutagenic effects of environmental contaminants.

US-Yugoslavia Cooperation

Under the auspices of the US-Yugoslavia Joint Board for Scientific and Technological Cooperation, NIEHS scientists continued collaborative studies in 1985 on the evaluation of the genetic effects of low levels of environmental chemical mutagens in bacterial systems, and comparison with eukaryotic cells.

Interagency Coordination

A number of federal and state agencies are involved in collaborative efforts to establish integrated systems for gathering, evaluating, and disseminating information on the health and environmental effects of chemical substances. The Assistant to the Director for International Programs represents NIEHS on the Toxicology Information Subcommittee (TIS) of the DHHS Committee to Coordinate Environmental and Related Programs (CCERP). This committee identifies the needs and establishes the mechanisms for the collection, storage, and dissemination of toxicologic information within DHHS. She is also the NIEHS representative on the Interagency Toxic Substances Data Committee (ITSDC) formed to design and coordinate an effective system for the retrieval of information on chemical substances submitted to EPA under the Toxic Substances Control Act; and a member of the Chemical Substances Information Network Subcommittee of the ITSDC which oversees the integration of a wide variety of data bases into one information network.

The Comprehensive Environmental Response, Compensation and Liability Act of 1980 (CERCLA), provides for several Federal organizations to participate in a coordinated response to provide information and advice on health hazards resulting from chemicals released into the environment and from the cleanup of hazardous waste disposal sites. In order to provide for the effective coordination of the collection, development and evaluation of the information necessary to determine the potential health hazards associated with such chemicals, the DHHS Committee to Coordinate Environmental and Related Programs established a Hazardous Waste Information Evaluation Subcommittee (HWIES) which is chaired by the Assistant to the Director for International Programs. The HWIES, composed of technical experts from various DHHS agencies, evaluates the available information on a number of chemicals frequently found in waste dumps and makes recommendations concerning the testing of these chemicals by the National Toxicology Program, and structured data record creation by the National Library of Medicine. The Assistant to the Director for International Programs is also a member of the Technical Advisory Committee of the Governor's Waste Management Board of the State of North Carolina.

In June 1985, the DHHS Committee to Coordinate Environmental and Related Programs sponsored a "Symposium on MPTP A Parkinsonian Syndrome Producing Neurotoxin". The Assistant to the Director for International Programs was a member of the Organizing Committee for this symposium.

PROGRAM PLANNING AND EVALUATION

OFFICE OF PROGRAM PLANNING AND EVALUATION
Summary Statement

During FY 1985 the Office of Program Planning and Evaluation (OPPE) continued planning, evaluation, program analysis, legislative support, and related activities. The most significant of these are summarized below. As in previous reports, these activities are summarized under two broad categories:

1. Areas of substantive program activity
2. Areas of functional activity

For the convenience of those who might wish to learn more about particular OPPE activities, the name of the staff member who worked on each is given.

Substantive Program Activities

Health Promotion and Disease Prevention (Ms. Hudson)

Throughout the year activity continued on the Toxic Agent and Radiation Control (TARC) prevention initiative, a part of the DHHS-wide program in health promotion and disease prevention. TARC is one of 15 Department established priority national prevention areas for improving the public health at the five major life stages. The Director of NIEHS was assigned primary responsibility for developing a DHHS-wide implementation plan and for coordinating activities regarding the Toxic Agent and Radiation Control goals through 1990. TARC agencies are CDC, FDA, HRSA, NIH, and NTP. Programs covered include lead toxicity, birth defects, use of x-rays, etc., and involve a variety of program mechanisms such as research, regulatory activities, and educational and informational programs.

OPPE responsibilities for this planning activity have involved providing staff support to the Director for the TARC initiative and serving as the lead agency contact with the Office of the Deputy Assistant Secretary for Health (Disease Prevention and Health Promotion) and the other participating agencies. During this past year OPPE planned and organized the TARC Work Group's Mid-Course Review (MCR) of progress and problems in implementing the 1990 Toxic Agent and Radiation Control prevention objectives for the nation. OPPE developed the status report for DHHS on TARC MCR activities, represented the TARC Work Group at a meeting of lead agency contacts on progress and problems with the mid-course reviews, developed the agenda for a subsequent TARC Work Group meeting to agree on goals and priorities, and coordinated review of a NCHS contractor-designed tracking system for the 1990 objectives and of the manuscript for the TARC progress review with the Assistant Secretary for Health (published January 1985 in Public Health Reports).

OPPE also coordinated Institute activities to develop material for other Departmental prevention activities. It prepared material for the Department's Prevention '84 publication, the DHHS occupational safety and health 1990 prevention implementation plan, and on the "Low Birth Weight Task Force" to ensure coordination of TARC and Pregnancy and Infant Health implementation plans.

National Toxicology Program (Mr. Kingman, Ms. Hudson)

During the year the National Toxicology Program (NTP) remained the focus of Congressional interest. OPPE continued to serve the Institute liaison with a GAO team charged by the Dingell Subcommittee with reviewing NTP contract procedures and related issues. During the same time OPPE coordinated development of NTP responses to a series of letters from the Congress which asked multiple complex question(s) concerning both administrative practices of NTP and substantive results of its research. The GAO review was completed in the Spring of the year and resulted in a report which commended NTP on steps it had taken to strengthen its testing programs.

Throughout most of the year OPPE continued to serve as liaison between NTP and the Agency for Toxic Substances and Disease Registry (ATSDR). This involved responsibility for negotiation of the Interagency Agreement which provided for transfer of Superfund money from ATSDR to NTP. In addition OPPE participated in development of the Memorandum of Understanding (MOU) between the PHS and EPA. Finally, OPPE coordinated development of monthly reports of NTP progress under Superfund.

Because NTP Superfund related activities had matured to the point that they could be considered operational, late in the fiscal year Mr. Kingman asked that NTP-ATSDR liaison responsibilities be moved out of OPPE to an office with operational responsibilities. This responsibility was transferred to Dr. Terri Damstra, Special Assistant to the Director for International Programs. This was a most appropriate collateral assignment as Dr. Damstra also chairs the Hazardous Waste Information Evaluation Subcommittee (HWIES) of the Committee to Coordinate Environmental and Related Programs. This subcommittee evaluates chemicals for Superfund-related toxicity testing.

OPPE also developed material on NTP for various Departmental purposes, including transition papers for the new Assistant Secretary for Health, NTP research accomplishments for the Secretary's briefing notes, and background material on NTP for the NIH Director's Advisory Council meeting.

Task Force III (Mr. Kingman)

In the 1983 House Appropriations Report, NIEHS was directed to develop a new Task Force to review progress in selected research areas in the environmental health sciences. The results of this Congressionally mandated effort will provide a guide for NIEHS research through the 1980's. The Task Force Report was transmitted to the Committee on Appropriations in January, 1985. Since that time OPPE staff has been working with the Task Force Co-Chairman to have the report printed by the Government Printing Office for broad-scale distribution. It is hoped that the report will be available in late 1985.

Functional Activities

Program Planning Activities (Mr. Kingman, Ms. Hudson)

OPPE continues to be involved in a broad range of planning activities. Among material prepared by OPPE staff are NIEHS Research Advances, NIEHS Overview for Congressional and visitor briefing books, NIEHS section of the NIH Modular Orientation Manual, and research accomplishments for the NIH Director's presentation before the House Science and Technology Committee. During the Year OPPE continued to carry out responsibility for developing the NIEHS annual program review with the Director, NIH and his staff.

Program Evaluation Activities (Ms. Hudson)

OPPE continued its involvement in a variety of program evaluation undertakings through coordinating activities funded through the "set-aside" evaluation program and preparing the annual NIEHS Evaluation Plan and managing its execution. At the end of FY 1984 OPPE facilitated award of the toxicology and epidemiology training program evaluation project contracts. These studies should take approximately three years each and, when completed, will assist in filling out the details of the manpower supply/demand situation for the environmental health sciences field.

OPPE staff also developed and provided continuing guidance for a major alteration in managing funding for the Institute's "set-aside" evaluation program; and developed information, guidance, and administrative procedures for management of evaluation study contracts. The program analysis system (PAS) evaluation study has been deferred until FY 1986 due to the resignation of the contract systems analyst and the necessity to recruit a government systems analyst.

Legislative Analysis Activities (Ms. Hudson)

As in the past OPPE staff continued to maintain a legislative library of pending legislation and relevant background material on a wide range of issues including animal welfare, clean air-acid rain, hazardous waste-superfund, occupational safety and health, pesticides, PHS issues, science education, technology transfer, toxic substances, and water pollution. This year OPPE made extensive use of LEGI-SLATE and the Congressional Quarterly's WASHINGTON ALERT SERVICE (computerized information services) to provide "Legislation of Interest to NIEHS" [alphabetical listing], weekly hearing schedules, listings of House and Senate leadership and members, congressional biographies, analyses of voting records, and analyses of bills of special importance to NIEHS (e.g., bills that mention NIEHS or NTP--superfund, clean air, occupational safety and health).

This baseline legislative effort supports specific Institute legislative analysis activities and is designed to keep the Institute Director and staff informed of legislative developments in Congress, to identify areas that may require Institute action, and to provide support for the Director's activities. During the year OPPE prepared analyses and briefing papers on policy issues and bills of critical importance or interest to NIEHS, including

the veto of NIH reauthorization bill, status of appropriation bills, introduction of various superfund bills, "Legislative Summary and Outlook", and bill provisions (e.g., PL 98-616 RCRA provisions relating to ATSDR, NTP, and HHS; six Florio toxic chemical bills; Bradley's S. 596 and Sikorski's HR 2022 superfund bills).

Program Analysis Activities (Ms. Hudson)

Throughout the year OPPE staff planned and carried out a variety of ad hoc program analysis assignments. Among these were:

- o Development and review of the FY 1983 Maternal and Child Health Inventory for NICHD
- o NIEHS Activities in Technology Transfer and Assessment for NIH's Office of Medical Applications of Research (OMAR)
- o National Cancer Program Director's Report and Annual Plan 1983-84 FY 1986-1990 for NCI
- o Catalog of Federal Projects in Marine Pollution Program, Marine Pollution Program: Agency Program Summaries, and Federal Marine Science Report 1984-85 for NOAA

Administrative Activities

This year brought VT220s into the lives of the program analysts. By year's end analysts were writing on the VAX and transmitting their drafts to the CPT. This has made editing a far simpler task and significantly reduced the amount of clerical time required to prepare documents. The coming years will undoubtedly bring increased dependence on computer-supported editorial capability.

Late in FY 1984, Mrs. Karen Hoffman announced her intention to resign in order to remain home with her children; she went on LWOP at the end of the calendar year and resigned from the staff in July, 1985. Mrs. Hoffman joined the OPPE staff in the summer of 1978 as a Presidential Management Intern. She developed quickly into a valued member of the OPPE staff, able to carry out a wide range of responsibilities, requiring continuing judgement and sensitivity to the implications of important program and policy issues. Her departure has left a critical gap in the OPPE staff.

While OPPE lost the services of its Legislative Analyst during FY 1984, it has been fortunate in having among its staff Ms. Gena Tolbert, who has assumed responsibility for using the LEGI-SLATE and CQ WASHINGTON ALERT SERVICE to provide legislative support services for the Institute. She has demonstrated remarkable initiative and resourcefulness in learning and applying these new systems. Ms. Willia Green, the OPPE stay-in-schooler, also has provided invaluable assistance and has proved to be a quick study with regard to new information systems.

Final Note

Early in the fiscal year, I (George Kingman, Director, OPPE) announced my intention to retire from Federal Service in the Spring of 1986. This will bring to a close almost thirty years of service on the staff of the National Institutes of Health and nineteen years with the National Institute of Environment Health Sciences. It has been a great privilege to have served throughout three decades of exciting developments in biomedical research, and almost two decades in the field of environmental health. This period has spanned the terms of seven Presidents. While the field of environmental health has matured greatly in the last twenty years, and moved center stage in the arena of science and public policy, like the poet I believe "the best is yet to come."

I owe a great debt to those with whom I have served over the years, and who have helped me so much in my many different assignments. I will miss them greatly.

FACILITIES ENGINEERING

OFFICE OF FACILITIES ENGINEERING

Summary Statement

The Office of Facilities Engineering (OFE) plans, directs, supervises and coordinates all facilities activities including, but not limited to, engineering design, inspection, construction, master planning, operation of utility plants and systems, maintenance and repair of all real property (buildings, grounds, surfaced areas, utility plants and systems), maintenance of facility operations equipment and vehicles, fire prevention and protection, custodial, refuse collection and disposal, supply and storage of construction and operations materials, and other miscellaneous facilities engineering services and operations.

At the direction of the Director, NIEHS, OFE performs non-facility engineering mission support work including, but not limited to, security, graphics, photography, and the design, fabrication, alteration and repair of intramural scientific instrumentation.

The work at NIEHS provides national and international leadership for similar installations throughout the world (grants and institutions), therefore, the facilities and equipment are in the forefront as models for use by research facilities outside of NIH, nationally and internationally.

Facilities:

OFE is responsible for the entire NIEHS facility comprised of 194,260 square feet of leased space, and 464,868 square feet of Government-owned space. The North Campus site consists of 154,274 square feet in a complex of 19 buildings, housing a staff in excess of 500 fulltime permanent employees.

The new NIEHS administration, laboratory, and support facilities, now complete, are on a 509-acre tract of land in the Research Triangle Park in close proximity to the North Campus facilities. These new facilities are now fully occupied. NIEHS will continue to occupy the North Campus facilities, while "off-site" leased facilities will be relinquished, except for warehouse space. The Extramural Program, presently located in the Burden Creek Facility on South Campus is to be relocated to the North Campus Facility.

Office Functional Sections:

OFE is divided into six functional sections under the Office of the Chief. The Facilities Management Section is the coordination point for all service requests providing planning/estimating, maintenance scheduling, material expediting, shops materials and parts storage and disbursement services to OFE. The Facilities Management Section maintains all work order, contract, and manpower management records. The Engineering Design Section provides architectural and engineering support required for planning new NIEHS facilities, improvements, major repairs and alterations through in-house design or contract with A/E firms. The group also provides architectural

and engineering support for the administration and inspection of NIEHS construction performed under contract.

The Operations Section oversees operations and maintenance of the power plant and building mechanical and electrical systems on the NIEHS South Campus. The power plant houses two 40-million BTUH oil, coal, and gas-fired boilers and two 2,500-ton water chillers. These systems are in continuous operation and deliver the environmental control (heating, cooling and humidity) to all facilities on the permanent site. Also, leased facilities operations are managed by this section in concert with the Facilities Management Section. The Maintenance Section provides shop services to the Institute. This includes 3 units: electrical, carpentry, and mechanical. Together these units respond to emergency repair calls, make minor alterations, and otherwise perform maintenance, major equipment preventive maintenance and repair.

The Instrumentation and Arts Section provides arts, graphics and photographic services to Institute personnel as adjuncts to publications of papers, conferences, seminars, and scientific exhibits. Additionally, the section provides instrumentation fabrication services and also supports repair and maintenance.

A new section, the Special Projects Section, was recently organized to coordinate planning, design and construction of an addition to Building 101 and related site improvements and utilities systems expansion on South Campus.

Goals and Accomplishments:

In Fiscal Year 1985, OFE directed a major effort toward completing design and construction of alterations to buildings on the North Campus. A major renovation was completed in Building 15. The Laboratory of Behavioral & Neurological Toxicology has moved a major part of its program into Building 15. Alterations are still going on in Building 6 to house the mass spectrometry group of the Laboratory of Molecular Biophysics. Alterations are underway in Buildings 2 and 3 to accommodate the Extramural Program. Construction will begin soon on alterations in Building 10 to house the Office of Administrative Management which currently occupies leased facilities near the Research Triangle Park.

Construction is in progress to replace the patio tile at Building 101 on the South Campus to eliminate serious construction flaws. Construction is also underway to repair faulty flashing and roofing on Building 101. Studies are underway to correct serious sound level problems in the air conditioning systems in the laboratories; and control problems on the air conditioning systems for the administrative areas.

OFE has begun to automate some of its administrative requirements. An automated file system for key and lock controls has been implemented. A program for procuring materials and services under the Record of Call method has been developed and testing and evaluation will begin soon. An inventory management system has been developed and there are various automated procedures established and ready for testing which will

complement the current work order system. OFE has accomplished these new procedures by contracting two program analysts and a data entry clerk through the GSA contract with the Institute Computer Technologies Branch. In addition, OFE has procured and taken delivery of a CPU as the base for the automated system. Other hardware and software are pending delivery and installation. Full operation is expected to begin by the end of the current fiscal year.

A Preventive Maintenance equipment inventory has begun and is going well. An award was made for this contract in December.

Future Branch Objectives:

During the next Fiscal Year, OFE will concentrate its efforts in the following areas: (1) completion of alterations in buildings on North Campus, (2) completion of a Program of Requirements and start of design for additions to program and support facilities (Building 101 addition), and (3) continuation of a contract effort to provide a comprehensive preventive maintenance program for all physical facilities and equipment on South Campus.

OFE recently awarded an Architectural/Engineering contract to develop a Program of Requirements for additions to Building 101 and related support facilities on the South Campus. A schedule has been established to prepare construction plans and specifications, award construction contracts and complete construction of the facilities in the spring of 1991. It has been the intent of NIEHS in its long range plan to complete facilities on South Campus to house all NIEHS activities on the one campus before the current leases expire on North Campus expire in May 1991. The newly organized Special Projects Section in OFE will direct all its attention to the completion of the facilities within the established time schedule.

Efforts will continue in the area of automation for more effective and efficient operation of OFE. The work order system is scheduled to be in operation within the coming fiscal year and OFE has the lead in working out the Record of Call procurement system for its application to the rest of the Institute. The Preventive Maintenance system requirements will be established as the equipment is inventoried and our analysts will be preparing recommendations for the software to fulfill that need. As the maintenance procedures are readied, following the inventory, many of those service requirements will be scheduled and contracts established to perform them. These procedures will significantly reduce the frequency and magnitude of breakdown repair at our facility.

HEALTH AND SAFETY

HEALTH AND SAFETY OFFICE Summary Statement

The NIEHS Health and Safety Office is administratively located within the Office of the Deputy Director and has broad responsibility for chemical and radiation safety, physical safety, fire protection, emergency preparedness, environmental protection and occupational health surveillance. The Health and Safety Office conducts research studies and special investigations relative to specific safety concerns.

Toxicological research laboratories pose unique health and safety problems due to the great variety of chemicals and radioisotopes that may be used and the non-routine nature of many laboratory procedures. The primary emphasis of the NIEHS Health and Safety Office is to minimize exposures and unsafe conditions through utilization of containment equipment, following appropriate work practices and procedures and use of personal protective equipment. The primary tools for accomplishing this objective are the required hazardous agent safety protocol, employee training and programs for information dissemination.

Programs for safe use of hazardous chemical agents are high priority at NIEHS. There were approximately 132 active protocols for use of hazardous chemicals in force during FY85. An important component of the Health and Safety Program is routine surveys of all laboratories, shops and warehouses on a quarterly basis. These surveys serve to identify potential hazards and to initiate preventive actions as well as to maintain an awareness of potential hazards. During FY85 a special survey of chemical storage practices was conducted and a set of guidelines developed and distributed to all Laboratories and Branches.

Use of primary containment devices and other engineering control measures is the preferred means of minimizing occupational exposures. All laboratory hoods, a total of 145, are inspected and their performance measured on a quarterly basis. Information concerning deficient hoods is immediately referred to the Office of Facilities Engineering for corrective action. In addition to fume hoods, a total of 46 biological safety cabinets are in use at NIEHS. All biological safety cabinets are tested and certified annually by an independent testing firm.

During FY85 the Health and Safety Office expanded and improved programs for monitoring occupational exposures. Routine sampling programs for organic vapors in laboratories, shops and waste processing areas were expanded both in frequency and extent of coverage. A special sampling program for formaldehyde exposures was completed and a routine formaldehyde monitoring program established. Other special air sampling studies completed included evaluation of wood dust exposures among animal bedding handlers and a study of potential trichloroethylene exposures during administration of this compound to test animals. A quality control program for the industrial hygiene laboratory was initiated which includes written procedures, preventive maintenance schedules for instrumentation, calibration procedures and recordkeeping requirements. As a part of the quality control program,

NIEHS applied and was accepted as a participant in the NIOSH Proficiency Analytical Testing Program (PAT) for organic solvents. The PAT program will provide a good means of quality control for analytical procedures.

The primary emphasis of NIEHS health and safety programs is exposure prevention through proper experimental design and use of laboratory containment equipment. Personnel protective equipment is used to supplement other preventive measures. A written respiratory protection program has been developed which includes initial selection criteria, qualitative fit testing, training of new users and annual maintenance checks by the Health and Safety Office. There were approximately 89 occasional users of respirators at NIEHS during FY85.

Use of radioisotopes and radiation sources has become an integral part of biomedical research. The Institutes' use of radioisotopes continues to increase with over 200 active protocols for the use of radioactive material. Approximately 1200 shipments of radioactive material were received in FY85 representing an increase of approximately 20% over FY84. The Institutes' use of ^{32}P has continued to increase while the use of other isotopes remained about the same as in FY84. In FY85 new procedures were established to expedite the ordering of isotopes while at the same time assuring that all radioactive material is properly accounted for.

Routine duties of the radiation protection program include monthly laboratory surveys, surveys of sealed sources, checking for contamination in cases of suspected spills, receiving and distributing incoming isotopes, calibration of radiation detection instruments, disposal of radioactive wastes, bioassay procedures, monitoring of personnel exposures and keeping an inventory of all radioisotopes at the Institute. In addition to these routine duties, special investigations are conducted to address specific issues relative to NIEHS radiation safety programs. A two year study on the fate of incinerated radionuclides was completed in FY85 and a manuscript submitted for publication. New studies were initiated on the dosimetry and calibration of beta emitting radioactive material. An extrapolation chamber was used in conjunction with National Bureau of Standards traceable beta sources to calibrate dosimeters and radiation detection equipment. This study will continue in FY86 and will evaluate the exposures of personnel handling beta emitters such as ^{32}P in various laboratory procedures.

The Health and Safety Office has responsibility for providing occupational health services for NIEHS employees. Services are provided through an interagency agreement with the PHS, Division of Federal Employee Occupational Health. Services provided include emergency treatment, periodic occupational health surveillance programs, preventive health programs, health promotion and education programs. During FY85 an automated occupational safety and health data management system was fully implemented and will provide integration of potential exposure information with health surveillance data. A special project to develop a surveillance program for laboratory animal allergies was initiated during FY85. The initial implementation included a questionnaire to determine allergy prevalence among NIEHS employees and initiation of an industrial hygiene study to identify potential exposures to airborne allergens.

during animal handling. In FY85 an Employee Assistance Program was developed and initiated. This program will provide employees with assistance in dealing with psychological or emotional problems potentially affecting work performance. Great care has been taken to assure confidentiality of all information from this program.

The Health and Safety Office continues to seek ways of improving the Institutes' hazardous waste management programs. During FY85, an additional full time on-site hazardous waste chemist was added to assure the timeliness of waste processing and to improve recordkeeping. The building design of a new 5000 square foot waste processing facility was completed in FY85 with construction to begin in FY86. An environmental impact assessment for this building was completed and approved.

Safety and health training is an important component of the Institutes' safety program. The Health and Safety Office offers a number of courses for laboratory personnel including "General Laboratory Safety," and "Introduction to Radiation Safety". Approximately 100 employees attend each of these courses per year. A course syllabus was developed for the General Laboratory Safety course. The syllabus will provide attendees with excellent reference material which will supplement the Institutes' Safety and Health Manual. In addition to the above laboratory safety courses, routinely scheduled courses in CPR, First Aid, and "Fire Extinguisher Use" are made available to all interested NIEHS employees. The Health and Safety Office also provides other special training as necessary. A one day course in "Biosafety Awareness" was sponsored jointly with the NIH Division of Safety in FY85 and had a total of 58 participants. Other special training provided in FY85 included instruction in proper lifting techniques for Comparative Medicine Branch personnel and use and care of compressed gas cylinders for warehouse personnel.

Safety and Health programs and training for NIEHS employees other than laboratory workers, such as shops and maintenance personnel, is an important area of emphasis. Major new programs including confined space entry and lockout/tagout procedures were developed and fully implemented during FY85. Among other requirements, the confined space program requires Health and Safety Office monitoring of all confined areas such as valve pits, electrical pits and tanks for hazardous conditions on a routine basis and before entry by maintenance personnel. Monthly safety training meetings for Office of Facilities Engineering personnel continued in FY85 covering a variety of topics.

Fire prevention and emergency preparedness continue to receive considerable attention. During FY85 the Occupant Emergency Plan for Building 101 was reviewed and updated and a plan developed for the warehouse and engineering complex. Smoke detectors were added to the elevator lobby in Building 101 and plans for a public address system completed. Several new fire hydrants were added for fire protection of NIEHS facilities. Safety and fire inspections of low hazard areas such as offices were expanded in FY85.

Responsibility for the Institutes' environmental protection programs reside with the Health and Safety Office. During FY85 a test burn of the large South Campus waste incinerator was completed. Test results demonstrated compliance with North Carolina air pollution regulations. A detailed survey

of all NIEHS storm drains and underground storage tanks was completed to assure compliance with all applicable ground water regulations. During FY85, a protocol for a detailed environmental audit was developed jointly with the NIH Division of Safety.

LIBRARY

LIBRARY AND INFORMATION SERVICES OFFICE
Summary Statement

The NIEHS Library is the principal science reference resource for the Institute. Library and information services include reference services, computerized literature searching of bibliographic and scientific databases, maintenance of a collection of 700 periodical titles and 13,000 books on environmental health, participation in a nation-wide network for interlibrary loan and cataloging, procurement of 1,450 new books for the Library and the laboratories, and publication of a monthly newsletter and the annual bibliography of publications by NIEHS personnel.

Reference/Literature Searching: The Library maintains one of the most advanced computerized literature searching capabilities in the world, with access to more than 200 databases covering subjects from toxicology to business administration. During FY85, Library personnel performed comprehensive multi-database searches on over 1,100 topics. Most of the year the Reference Librarian position was vacant, but during one three-month period 700 reference questions were answered. The most heavily used databases continued to be TOXLINE, MEDLINE, Toxicology Data Bank, Biological Abstracts, and Chemical Abstracts. Examples of search requests include the following: comprehensive literature search on methyl isocyanate for the International Office to respond to the Bhopal, India, emergency and for TRTP to begin testing; comprehensive search on other recent chemical emergencies for background briefing for Dr. Rall; comprehensive searches on the relationship of high altitudes and cancer and on leukemia in refinery workers for BRAP; searches on porphyrias and chemicals and on naturally occurring toxicants in food; and numerous searches for toxicological information on chemicals for TRTP. Library staff also began a project to provide training to scientists who want to do their own computerized literature searches. Eleven TRTP Chemical Managers were given the initial training in the use of toxicology databases.

Journal Collection: The journal literature continues to be the primary means of disseminating scientific information. The Library subscribed to approximately 700 periodicals during FY85, and, in addition, ordered 400 subscriptions for the various laboratories. The Library continued to bind journals selectively or replace them with microfilm to save space. The collection now includes 20,000 journal volumes and 1,750 microfilm reels. The Library, working with the EPA Library, updated its computer-generated journal holdings list for FY85.

Book Collection: Continuing the development of the book collection, the Library ordered 1,450 books in FY85, of which 40% were ordered for the Library and 60% for the laboratories. The Library also ordered more than 300 technical reports.

Computer Catalog: FY85 was the third year of operation for the C.L. Systems LIBS 100 computerized catalog and circulation system. This computer supports an online catalog of the books in the Library and in the Labs and is searchable by author, title, or subject using terminals in the Library or any terminal in the labs or offices, thus making it much easier for Institutes scientists to find out what books are in the Library. The system also speeds up the check-out procedure, produces overdue notices, and provides statistical reports for management purposes. During FY85 the system was used to check-out 250 books per month. An acquisitions software module was used to put all book-ordering

information on the computer and have purchase orders printed out. The LIBS 100 System was demonstrated to several library groups, including graduate students from the UNC School of Library Science.

The Library continued using the automated cataloging system, OCLC, a computerized union catalog of books held by more than 3,500 libraries nationwide. The NIEHS Library has experienced a tremendous savings in time owing to the 95% hit rate for new books which already have cataloging data on OCLC. Through an interface, catalog records are transmitted from the OCLC computer in Ohio to the LIBS 100 computer in the NIEHS Library where they are immediately integrated into the public catalog.

Interlibrary Loan: The number of photocopy and loan requests increased by 22% in FY85, the total being 19,500. For the third year in a row, more of the requests were filled from the Library collection (58%) than from other libraries through interlibrary loan (42%). This reflects the ongoing improvement in the NIEHS collection.

The OCLC computerized catalog also proved useful for verifying titles for interlibrary loan and for locating libraries from which to borrow books throughout the U.S. The NIEHS Library provided 527 loans or photocopies to other libraries or to individuals in the Research Triangle Park area. This 15% increase over the previous year reflects the growing importance of the NIEHS Library as a national resource.

Institute Manuscripts and Bibliography: The Library continued to maintain the NIEHS archives of manuscripts submitted for publication and list them in the monthly newsletter. More than 500 manuscripts were written by NIEHS scientists during the year. The Library published the 1984 NIEHS Bibliography, a catalog of the papers published by Institute personnel since 1966.

Experimental Data Repository: The Library continued the procedure of having laboratory notebooks microfilmed for archival purposes.

Planning and Meetings: Staff office space was remodeled and an office was converted to an environmentally controlled room for the computer. The three reading rooms in the E module of the South Campus laboratories were maintained with limited resources. At the request of OFE, the architect's plans for building a central library in A module were reviewed.

Close contact with various library and information organizations was maintained in FY85. Mr. Robertson served on the Library Services and Construction Act Advisory Council to the N.C. State Library, the body responsible for the distribution of federal funds to N.C. libraries. He was active in the Special Libraries Association on both the national and state levels, representing NIEHS at the national meeting in Winnipeg, Canada, and at the quarterly meetings in North Carolina. Mr. Robertson hosted the N.C.S.L.A annual workshop on "Creativity and Leadership." He was appointed Chairman of the N.C. Chapter's Government Relations Committee for 1984-85, and he was elected Secretary of the Chapter for 1985-86. Mr. Robertson also represented NIEHS at the annual Federal Field Librarians meeting in Washington, D.C., and at the fall and spring meetings of the C.L. Systems User Group. He chaired the Program Planning Committee for the UNC Library School Alumni Day.

Ralph Hester represented NIEHS at the annual Online Meeting in San Francisco.

INTRAMURAL RESEARCH PROGRAM

OFFICE OF THE SCIENTIFIC DIRECTOR

LABORATORY OF BEHAVIORAL AND NEUROLOGICAL TOXICOLOGY

LABORATORY OF BEHAVIORAL AND NEUROLOGICAL TOXICOLOGY
Summary Statement

The scientific efforts of the Laboratory of Behavioral and Neurological Toxicology (LBNT) are directed toward the understanding of the behavioral and neurological effects produced by toxic substances. Special emphasis is placed upon the changes in behavior and neurologic function produced by long-term exposure to low levels of a wide variety of chemical and physical agents present in the environment and to exposure during the development of the nervous system.

The goal of the Laboratory is to obtain a better understanding of neuronal plasticity and nervous system mechanisms responsible for adaptation. This goal reflects our opinion that environmental agents can act to interface with mechanisms permitting normal plasticity and/or adaptation. General approaches include the following: (1) investigations of the biophysical and molecular biological properties of excitable cells, (2) studies on afferent and feedback mechanisms which influence neuronal plasticity of selected functional systems, (3) studies on the neurobehavioral mechanisms involved in plasticity and adaptive responses to environmental agents, (4) studies on peptides and their effects on biological systems, (5) identification of vulnerable neural circuits by study of neurotransmitter translocations and enzyme analysis, and (6) studies on the functional roles of neurotransmitters or neuromodulators (such as brain peptides) in CNS adaptation following chemical or physical insult.

The research goals of the Laboratory are supported by both intramural scientists and contracted research. The scope of the overall effort is broad involving national and international programs and adjunct appointments. Training opportunities exist for graduate students at nearby universities, foreign and American postdoctoral scientists, and American scientists on sabbatical leave. Current expertise is in the areas of behavior, neuropharmacology, neurochemistry, neuropeptides and neurophysiology.

MEMBRANE PHYSIOLOGY SECTION

Neurophysiology of Adaptive Mechanisms Group

The ultimate goal of the Membrane Physiology Section is the understanding of neuronal plasticity and nervous system mechanisms responsible for adaptation to the environment. This is of particular relevance to toxicology since it is the interference with adaptive mechanism which gives rise to the signs and symptoms produced by a toxicant. Two general approaches are used. These are (1) investigation of the biophysics and molecular biology of excitable cells, and (2) studies on the afferent and feedback mechanisms influencing neuronal plasticity of selected functional systems and their alteration by known or putative stressors. Perturbations in these systems are induced using selected neuroendocrine, neuropeptide and organometal compounds, and non-ionizing electromagnetic radiation. Each class of agents has members which have been implicated in producing alterations in neuronal excitability. Comparison of the effects produced by these agents should provide a better understanding of the phenomena studied and insights into the mechanisms whereby the agents produce their effects on excitable tissue.

Some members of the membrane physiology section are investigating the relationship between synaptic activity and the development of sensitive to excitatory and inhibitory aminoacids in culture of a mouse spinal cord neurons. It has been found that at days 2-3 in culture, spinal cord neurons are sensitive to quisqualate, glutamate and γ -amino-butyric acid. However, response to kainate are small or absent. By day 7 in culture, spinal cord neurons are highly sensitive to all agonists tested. This increase in sensitivity to the amino-acids coincides with the development of spontaneous synaptic activity.

Aspects of seizure activity induced by pentylenetetrazol and electroconvulsive shock, and their alteration by neuropeptides and organometal compounds are also being investigated. This work is being done in collaboration with members of the neuropharmacology section. With respect to organometal compounds, work in this and other laboratories has provided evidence that the limbic system is a vulnerable site for effects of at least some organometal compounds. Recent studies have demonstrated that triethyl lead, in particular, markedly accelerated the kindling of pentylenetetrazol-induced seizures. The assessment of the neurophysiological bases for pentylenetetrazol-induced kindling and the mechanisms whereby triethyl lead exacerbates it should further our knowledge of excitatory phenomena of the nervous system and how these processes can be altered by neurotoxic substances.

Non-Ionizing Radiation Group

The Non-Ionizing Radiation Workgroup has studied the biological effects of 2.45 GHz microwave radiation during the past year. In vitro and whole animal biological systems have been utilized in order to study both the basic interaction mechanisms of microwaves with biological material and the overall effects on more complex specimens i.e., the whole animal. In vitro preparations used have included rat atria, frog sciatic, and lobster giant axons. Frog sciatic nerves have been exposed to continuous wave (CW) and pulse microwave radiation. Rates of fatigue or loss of vitality (the ability of the nerve to continue firing under rapid stimulation) was increased in the nerve exposed to 2.45 GHz at a specific absorption rate of 7 mW/g but not at 4 mW/g. This result suggests that the nerve vitality is nonlinear with respect to microwave intensity. This type of nonlinear behavior would be expected if the neural membrane is acting as a diode-like detector of the microwave field. In order to study this membrane interaction and to obtain a basic understanding of the ionic transport and gating mechanisms in excitable membranes, giant axons of the lobster ganglia are now being studied.

The influence of microwave radiation on cardiac tissue using in vitro and in vivo methodologies was also studied. A method for exposing isolated rat atria to microwave radiation has been developed. The data suggest that 2.45 GHz CW microwave radiation of 2 or 10 mW/g has no overt effect on the rate of force of contraction of isolated atria. In addition, the response of atria to drugs was not influenced by microwave exposure. Specifically, the dose response curve for isoproterenol and acetylcholine was not influenced by either 1, 10 or 100 mW/g exposure and the ability of propranolol and atropine to inhibit the isoproterenol and acetylcholine response of rat atria was not altered by these exposure levels. Also, certain biochemical and physiological parameters, which are indicative of cardiac integrity, have been measured in unanesthetized rats during whole body ventral exposure to 24-50 MHz CW microwaves. The data suggest microwave exposure of 10 mW/cm for 6 hr has no effect on mean arterial blood pressure or colonic temperature. However, there was a microwave induced bradycardia which was exhibited after 30 min of microwave

exposure at 10 mW/cm^2 and ambient temperature of 22°C . The bradycardia persisted throughout the remainder of the 6 hr exposure period. None of the biochemical or hematologic indices examined were influenced by this exposure level. When rats were exposed to the same microwave radiation in an ambient temperature of 27°C , no change in heart rate was observed. However, with the same microwave radiation and the ambient temperature at 30°C , an increase in heart rate (tachycardia) was measured. This work indicates that microwave radiation of 2450 MHz frequency does not have a direct effect on the pacemaker cells of the heart, but that the cardiovascular system of the rat responds with metabolic changes due to the energy absorbed to maintain homeostasis. Therefore, depending on the intensity of the microwave field and the environmental conditions (temperature and humidity), bradycardia, no change or tachycardia might be produced by the microwave exposure.

Research has been conducted to determine the effects of exposure to microwave radiation during embryonic development under a contract with N.C. State University. The research uses fertile Japanese quail eggs. These studies are designed to determine the response of quail exposed during development to stressful conditions at juvenile and adult ages. In all cases the exposures were at a microwave frequency of 2.45 GHz, at an incident power density of 5 mW/cm^2 (specific absorption rate = 4.03 mW/g) and during the first 12 days of development. The hematological response of immunized quail was measured in juvenile (5 weeks old) and adult (22 weeks old) quail at 3, 6, and 9 days post-immunization. Only the exposed juvenile female quail elicited a response different from controls. Total reticulocytes were initially depressed and, at 9 days post-immunization, they expressed an increased number of reticulocytes in comparison to controls. The response of quail exposed to microwave radiation in the above manner to hemorrhagic stress also was investigated. The quail were mechanically hemorrhaged to 70% of their calculated blood volume. Circulating erythrocyte and reticulocyte numbers were depressed below controls by 24 hr post-hemorrhage in exposed juvenile and adult females. Lymphocyte numbers were depressed below controls by 24 hr post-hemorrhage in exposed juvenile males at 72 hr. These data suggested that hematopoiesis is affected by embryonic irradiation and the effect persists for a long time after hatching (exposure).

The research completed during the past year contributes to the program of LBNT by providing information on the biophysical interaction of an environmental agent with excitable cells. In addition the results help to explain how animals respond, adapt and compensate to a combination of stressors either simultaneously or sequentially imposed. A better understanding of the response of the biological systems and the manner in which the biological systems alter their normal function when exposed to non-ionizing radiation has been obtained.

NEUROBEHAVIORAL TOXICOLOGY SECTION

Neural Mechanisms Group

Behavioral and neurologic alterations in response to environmental agents often represent the earliest manifestations of toxicity in animals. The research program in the Neurobehavioral Section is dedicated to the study of the behavioral and neural effects of environmental agents and to the elucidation of their mechanism of action. In addition, the program is concerned with the compensatory or adaptive processes activated following exposure to these agents. Information derived from such research may be useful in the development of strategies to treat

exposed individuals as well as providing a basis for understanding environmentally related neurological diseases.

The neuropharmacological basis for organochlorine-induced tremor and hyperexcitability was studied in rats. Both chlordecone and p,p'-DDT increased the release of brain norepinephrine and serotonin, while having marginal effects on dopamine; p,p'-DDT, but not chlordecone, was found to increase tissue levels of excitatory amino acids such as aspartate and glutamate in the brain stem and spinal cord. Pharmacological experiments to determine the functional significance of these neurochemical changes showed that cholinergic and serotonergic receptor antagonists attenuated the tremor produced by chlordecone, but enhanced that produced by p,p'-DDT. Blockade of α -noradrenergic receptors attenuated tremor produced by both organochlorines. Previous studies showed that pretreatment with the anticonvulsant phenytoin attenuated the tremor produced by p,p'-DDT, but enhanced that produced by chlordecone. Recent work extended this observation to augmentation of acoustic startle response produced by p,p'-DDT and chlordecone. Permethrin, a Type I pyrethrin believed to have the same mechanism of action as p,p'-DDT, was found to have virtually the same neurochemical effects as p,p'-DDT; pretreatment with phenytoin also attenuated the neurological effects of permethrin. Intraventricular administration of calcium prior to the administration of p,p'-DDT or chlordecone attenuated or enhanced the tremorigenic effects produced by these agents, respectively. These experiments demonstrate the neurological manifestations produced by many of the organochlorines, such as tremor and augmented startle responsiveness, are similar, suggesting that they may activate a final common pathway; however, the neuropharmacological basis for the effect may be different.

Alkylmetals were studied for their potential to produce selective neurobehavioral and neurochemical alterations in limbic forebrain function. Short-term repeated or acute exposure to triethyl lead (TEL) impaired passive avoidance and facilitated two-way avoidance, effects similar to those observed in animals with limbic forebrain lesions. However, subsequent work indicated that TEL did not affect working memory as determined by performance in the radial-arm maze. Comparisons of trimethyl lead (TML) with TEL indicated that they produce quantitatively different behavioral effects, possibly due to toxicokinetic factors leading to differential neuromorphological changes in the CNS. Neurochemical studies indicated that TEL, TML and triethyl tin (TET) had few specific effects, but trimethyl tin (TMT) produced a pattern of neurochemical changes indicative of hyperammoniaemia. Although it is evident that TEL is not a specific limbic toxicant, other experiments demonstrated that it did produce specific alterations in neurobehavioral function (antinociception, increased responsiveness to pharmacological challenge) related to corresponding changes in neurochemical function (receptor binding, increased enzyme activity). Experiments with other potential limbic toxicants such as intracerebral administration of AF64A, a cholinergic cytotoxicant, produced signs of limbic forebrain dysfunction (impaired passive avoidance and radial-arm maze performance and increased motor activity); these effects were associated with 50% decreases of acetylcholine (ACh) content in the hippocampus and frontal cortex. Destruction of granule cells in the dentate gyrus with colchicine produced similar behavioral effects. These studies indicate that cytotoxicants such as AF64A and colchicine may be useful in the study of the neurobiological basis of neurodegenerative diseases involving limbic forebrain function.

One common finding in all of the research conducted by this group is that the nervous system demonstrates considerable resiliency or plasticity following exposure

to environmental agents. In one respect, this is important since subtle structural or functional damage may be present in the absence of obvious toxicological endpoints. Examination of behavioral and neural function under appropriate conditions frequently reveals the extent and nature of these alterations. On a more basic level, the compensatory and adaptive processes that are activated by exposure to environmental agents represent a basic feature of neural activity. The study of such processes using neurotoxicants as tools may lead to a better understanding of the functional integration of the nervous system.

Developmental Neurobiology Group

The maturation of the central nervous system and its functional output, behavior, reflect a series of highly ordered and precisely timed events. The consequences of early perturbation of behavioral and neural development reflect a dynamic interplay between the greater susceptibility of the immature organism to environmental and chemical insult and the greater capacity for plasticity and reorganization in the immature nervous system. The study of perturbations of this dynamic balance and how they are reflected in recovery or sparing of function (or a lack thereof) are important to elucidation of the processes controlling appropriate behavioral and neural development. Specifically, the developmental neurobiology workgroup has focused on perturbations of the developing hypothalamic-pituitary-adrenal axis and the hippocampus induced by early exposure to various classes of environmental agents (chlordecone, triethyl lead, and carbon monoxide).

While neonatal exposure to chlordecone, an organochlorine insecticide, produces a similar characteristic profile of neurotoxicity to that following exposure in adulthood, longitudinal and time-sequential studies have indicated long-term alterations in body weight regulation, catecholaminergic and serotonergic function, and memory retrieval processes. The association of these chlordecone-induced alterations with functional imbalances in circulating and adrenal steroids, as well as rapid and apparently permanent changes in adrenal physiology, are consistent with the hypothesis that these changes in behavioral and neural function are in part attributable to neurohormonal activity and dysfunction of the hypothalamic-pituitary-adrenal axis.

Neonatal exposure to triethyl lead, the active metabolite of leaded gasoline, has been shown by quantitative neuromorphometry to produce a preferential and permanent loss of pyramidal cells of the hippocampus. This insult also results in a permanent increase in behavioral reactivity which is independent of sensory modality, early undernutrition, and early testing effects. The use of pharmacological probes and receptor binding techniques linked this enhanced reactivity to dysfunction of cholinergic, but not dopaminergic, connections with the hippocampus proper. In sum, the similarity of these observations to those following septal-hippocampal damage suggest that triethyl lead functions as a preferential limbic excitotoxin.

Prenatal carbon monoxide exposure was studied with maternal carboxyhemoglobin levels within the range experienced by human cigarette smokers. In our initial studies, disruption of hippocampal function was suggested by an impairment in the acquisition and retention of a two-way avoidance response in juvenile aged offspring of carbon monoxide-exposed dams. Multiple dependent measures and specific control groups confirmed that this deficit was independent of nonassociative and

motivational alterations. However, a marked attenuation of these associative impairment was observed upon maturation to adulthood. Additional time-sequential studies nevertheless indicated that such recovery of function was transient; with continued aging there was a pronounced exacerbation of learning and memory dysfunction.

NEUROPHARMACOLOGY SECTION

Neurotransmitter Mechanisms Group

The primary goal of the Neurotransmitter Mechanisms Group is concerned with the neurochemical and neuropharmacological bases for the effects of psychoactive compounds and neurotoxins on behavioral and neurological function. The major research effort has focused on the chemical modulation of neuropeptides and neurotransmitters. The major psychoactive compounds studied are dopamine-related agents, such as haloperidol. Seizure-induced changes of brain opioid peptides are also studied after electroconvulsive shock (ECS) or kainic acid (KA) treatment. The major neurotoxins studied are organochlorine insecticides, such as DDT or chlordane.

For the last few years, further progress of this study was hampered by the lack of a method to measure the turnover of opioid peptides. Our previous studies have been limited to the measurement of steady state concentrations of the peptides by radioimmunoassay (RIA). Since the steady state concentration does not precisely reflect the dynamic changes of peptide-containing neurons, development of turnover methods for opioid peptides is crucial for further understanding of the regulation of peptides. We have developed methods for measuring mRNA coding for enkephalin precursor (preproenkephalin A) as an index for the rate of biosynthesis of enkephalin. These two methods are cell free translation and blot hybridization using cDNA clone coding for preproenkephalin A. The main purpose of this project is to examine the molecular mechanism underlying the modulation of opioid peptides by haloperidol, ECS and KA. Repeated injections of haloperidol caused a two-fold increase in the striatal concentration of enkephalin. This increase was accompanied by a two-fold increase in the level of mRNA coding for the precursor of enkephalin. This suggests that haloperidol accelerates the turnover of enkephalin. Furthermore, this study demonstrates that long-term treatment with haloperidol affects the gene expression of the enkephalin system. This finding raises an important consideration that gene expression may be the ultimate site of action for antipsychotic drugs. Similar to haloperidol, repeated ECS also increased the brain concentration of enkephalin and level of mRNA coding for preproenkephalin A. This finding lends further credence that gene expression may be a common site of action for various psychiatric treatments. A single injection of DA caused recurrent seizure and produced a three-fold increase in enkephalin concentration in the hippocampus 72 h post-dose. This increase in peptide level was preceded by a large increase in the abundance of mRNA coding for preproenkephalin A. This study suggests that the hippocampal enkephalin-containing neurons are responsive to the seizure activity induced by ECS or KA. For future studies, we plan to use the newly developed cell free translation and blot hybridization methods to study the biosynthesis of enkephalin after haloperidol or ECS in greater detail. These studies should provide further information regarding the possible role of enkephalin in mediating the actions of haloperidol and ECS.

One of the most severe and consistent neurological symptoms observed in laboratory animals exposed to organochlorine insecticides, such as DDT or chlordane, was tremor. So far, very few neurochemical effects of these insecticides have been reported. Elucidation of neurochemical mechanisms underlying DDT and chlordane-elicited tremor can not only help us to understand the neurotoxicity of these insecticides but it may also provide further insight into the central control of tremor.

p,p-DDT and related agents act to hold the sodium channel open once opened and this effect is believed to be responsible for neurological effects of tremor and hyperexcitability in vivo. Tremor was almost completely blocked in rats pretreated with hydantoin, an anticonvulsant believed to block repetitive firing of nerves by interfering with the inactivation gates of sodium. A similar antagonism was observed for permethrin, a Type I pyrethroid believed to have a mechanism similar to that of DDT. However, hydantoin increased the tremorigenic effects of chlordane, an organochlorine whose mechanism has not been linked to the sodium channel. Our data are consistent with the hypothesis that in vivo neurotoxicity of some organochlorine insecticides is related to their effects on the sodium channel.

Neurochemistry Group

The major goals of the Neurochemistry Workgroup are: a) determining the biochemical basis for the effects of environmentally prevalent agents on behavior and neurological function, and b) identifying the primary targets of toxic agents and the sequence of events following initial damage by such agents.

Two major directions are being pursued to achieve these goals:

1. The regulation of intraneuronal calcium.

The concentration of intracellular free calcium within nerve cells is about 10^{-5} times that of extracellular calcium. The maintenance of this steep gradient is essential for cell viability since increased levels of calcium within the cell are rapidly lethal. Furthermore, abnormal levels of ionic calcium can disrupt specific neuronal functions including axoplasmic transport and neurotransmission.

Our work inquires as to whether the neurotoxicity of certain classes of chemical (organic metal derivatives and organochlorine insecticides) may be underlain by disruption of cell membranes leading to failure of normal calcium homeostasis. We will also examine the possibility that the transient elevation of synaptosomal calcium effected by depolarizing conditions, is mediated by biogenic polyamines. The following findings have been made:

- a. A system has been developed whereby ^{45}Ca penetrance into synaptosomes can be assayed within 2 seconds of depolarization. This depolarization-induced uptake is over 3 times as great as basal influx.
- b. Chlordane has no effect on depolarization-related Ca^{++} entry but the rate of background uptake is significantly elevated.

- c. Two recently available fluorescent compounds used for intracellular Ca^{++} assay have been compared for their appropriateness for synaptosomal preparations. FURA has been shown to be free of a variety of artifacts found with its predecessor QUIN 2. The calcium content of the synaptosomes that we use, responds as expected to depolarization induced by a variety of means.
 - d. Both chlordecone and methylmercuric chloride elevate intrasynaptosomal calcium in a prolonged manner from its basal value of around 500nM.
 - e. Putrescine causes a rapid and transient elevation of synaptosomal Ca^{++} . For such rapid changes, QUIN 2 is better used than FURA, since it is assayed at a single wavelength.
 - f. Putrescine has also been shown to facilitate depolarization-induced aspartate release; further evidence of its role as a mediator between events are the plasma membrane and changes in intracellular Ca^{++} content.
2. Ornithine decarboxylase (ODC) in the nervous system.
Polyamines have been associated with cell proliferation but their role in a relatively non-dividing organ, the mature brain, is little understood. However, ODC can be rapidly induced in the brain in response to a variety of stimuli.

A goal of this work is to increase understanding of the function of ODC in brain tissue.

A change in the chemical or environmental milieu or an organism frequently results in biochemical changes in many tissues. The distinction between primary and secondary changes is often unclear in view of the many adaptive responses an organism is capable of. The initial site of action of many "neurotoxic" agents may be at non-neural sites in many cases. In view of the complex brain-body interactions, it is often difficult to dissect out a chain of events following changes in environmental or chemical exposure.

This work is also directed toward pinpointing those tissues or brain regions that are initially affected by chemical agents or changed environmental surroundings. The approach is intended to permit a sequential resolution of reactive changes in response to various forms of disruption of normal homeostatic processes in the brain. ODC assays will be exploited as a tool in this study. Findings made this year include:

- a. A new form of particulate ornithine decarboxylase (ODC) in brain has been described and characterized.
- b. Cerebral ODC is elevated in the brain following electroconvulsive shock. This has been temporally and regionally delineated and related to circulating corticosterone levels and adrenal ODC.
- c. DDT and chlordecone dramatically elevated adrenal ODC. Since this effect also is seen in hypophysectomized rats, a direct effect on adrenals is implied. This has been further suggested in studies using a tissue culture preparation of adrenal cortical cells.

- d. The administration of the ODC inhibitor, difluoromethyl ornithine (DFMO) can prevent chlordecone-induced tremor. This blockage is overcome by simultaneous injection of putrescine. This is the first report of a behavioral effect caused by systemic injection of the carcinostatic DFMO, suggesting a distinctive neural role for polyamines.

Our current intent is to study the potential value of this enzyme, ODC, in delineating sites of responses of the nervous system to various environmental and chemical stimuli. ODC is rate limiting in polyamine synthesis and is an indication of cellular activation. We wish to test its applicability in delineating perturbation of ongoing metabolism in brain regions. For example, ODC will be assayed in both hippocampi following unilateral hippocampal lesioning with colchicine or kainic acid. The utility of ODC is likely to be especially large since:

- a. It can be elevated 20-50 fold in activated tissue and thus is likely to be very sensitive to low levels of perturbants.
- b. It has the shortest known half life of any protein (around 10 min) and thus its level can change very rapidly in responding tissues.
- c. It is found in all tissues. This allows the tracing of the temporal sequences of disturbances following changes to the chemical or physical environment of an organism.

The biochemical studies of the Neurochemistry Workgroup emphasize the detection of responses to environmental changes. Such responses can help to explain both the sites of action of toxic chemicals and the resulting behavioral alterations. In this manner the work reflects the laboratory emphasis on neuronal plasticity and cerebral compensatory processes.

Peptide Neurochemistry Group

This group focused on the isolation of putative neuropeptides from mammalian tissues that had sequence homology to amphibian peptides. They used highly specific immunological assays for the detection of the peptides coupled with a variety of chromatographic methods. They eventually purified 2 peptides, called PHLIPs, from rabbit stomach based on their cross-reactivity with an antibody directed toward the amino-terminal region of physalaemin. The sequence was deduced by application of fast-atom bombardment-tandem mass spectrometry using a computer program specifically designed to match the various fragments. One, an octapeptide, had the following sequence: pGlu-Val-Asp-Pro-Asn-Leu-Gln-Ala; the sequence of the other peptide, a heptapeptide, was identical with the exception of the carboxy-terminal Ala residue. Residues 1, 3-5 were homologous to physalaemin. The synthetic peptides cross-reacted with the physalaemin antibody to an extent of 1.5-2.5% in a non-parallel manner relative to physalaemin. Preliminary studies on their pharmacological bioactivity were inconclusive since any effect observed was in the nanomolar range rather than the picomolar amounts observed with most neuropeptides. Further experiments are in progress to determine if those peptides have a direct effect on other physiological and pharmacological parameters.

The other major area under investigation concerns the bombesin-like immunoreactive peptide (BLIP) from milk. A large scale isolation method was devised, but

unacceptable losses of peptide occurred which led them to modify their purification scheme in order to obtain microgram quantities of peptide. One crucial step in their new approach involves the synthesis of an immunoaffinity column which will be tested shortly. The peptide exhibited the characteristic bioactivity of bombesin on several tissues in vitro. A model system was also developed in order to test the purified and synthetic milk bombesin: the effect on gastric secretion by bombesin-like peptides in rats. Those effects, using various analogues of bombesin, determined that the bioactivity in that in vivo system required a tryptophan residue at position 8 and involved glutamine and histidine residues at positions 6 and 12, respectively. Modification at those sites led to marked decrease in bioactivity.

The goals of this group are to understand the mechanism(s) of action of their new peptides. One physiological approach involves a concerted effect of the synthetic peptides and monoclonal antibodies to the peptides on neuronal development. The tissue origin of the milk bombesin peptide and its physiological regulation remains an interesting but unanswered question. The presence of a Visiting Fellow in the group for FY-86 will coordinate that project with the biochemical data and interface their studies with other groups in the laboratory.

PERSONNEL

Additions to the Laboratory were: Visiting Fellow - Dr. A. Guglietta; Visiting Fellow - Dr. H. Komulainen; Visiting Fellow - Dr. S. Li; Visiting Associate - Dr. K. Saitoh; Staff Fellow - Dr. S. Sivam; Guest Worker - Dr. K. Takeuchi. Individuals leaving the Laboratory were: Expert - Dr. P. Chen; Visiting Fellow - Dr. T. Kanamatsu; Visiting Fellow - Dr. Y. Nakata; Staff Fellow - Dr. T. Walsh.

OTHER ACTIVITIES

Dr. S. C. Bondy: Adjunct Associate Professor, Department of Pharmacology, University of North Carolina, School of Medicine; Member, NIEHS Radiation Safety Committee; Member, Publications and Education Committee, American Society for Neurochemistry; Member, Editorial Board, Environmental Health Perspectives; Member, Editorial Board, International Journal of Developmental Neuroscience; Member, Editorial Board, Neurotoxicology, Member, Editorial Board, Neurochemical Research; Member, Organizing Committee of the Winter Conference on Brain Research; Invited faculty at NATO Advanced Study Institute on "Toxicology of the Nervous System," Belgirate, Italy, September 1984; Invited speaker at Symposium of Environmental Neurotoxic Substances, Riva del Garda, Italy, May 1985; Ad Hoc Reviewer, National Science Foundation (Neurobiology Program), Brain Research, Journal of Neurochemistry, Proceedings National Academy of Science, U.S.

Dr. J. S. Hong: Adjunct Associate Professor, Department of Psychiatry, Duke University Medical School; Adjunct Associate Professor, Toxicology Curriculum, University of North Carolina; Invited seminar entitled "Modulation of Brain Opioid Peptides by Antipsychotics and Electroconvulsive Shock," Medical College of Virginia; Invited seminar entitled "Hippocampal Opioid Peptides and Seizure Activities," University of North Carolina; Invited speaker of the Symposium entitled "Neural Membranes As A Target Of Toxicity," Finland; Invited speaker of the Symposium entitled "The Use Of Organochlorine Insecticides In Neurobiology," ASPET Meeting; Ad Hoc Reviewer for Brain Research, Science, Biochemical Pharmacology,

Neuropharmacology, Neurotoxicology, Toxicology and Applied Pharmacology, grant proposal from National Science Foundation (Neurobiology Program).

Dr. L.H. Lazarus: Adjunct Associate Professor, Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill; Member Linberger Cancer Research Institute, University of North Carolina, Chapel Hill; Ad Hoc Reviewer for Analytical Biochemistry, Cancer Research, Chemico-Biological Interactions, Journal of Biological Chemistry, Proceedings of the National Academy of Science, and Science; Invited guest speaker at the International Symposium on "Nonmammalian Peptides", Rome, Italy, May, 1985, and delivered paper entitled "Evolutionary relationship between nonmammalian and mammalian peptides", chaired the session "Nonmammalian Peptides: Amphibia, Birds and Reptiles", and co-authored two other papers at the symposium, "Central neuromodulation of gastric secretion by bombesin-like peptides", and "Active peptides from amphibian skin are also amphibian neuropeptides".

Dr. C. F. Mactutus: Membership accepted to the Psychonomic Society and the New York Academy of Sciences; Recognized in Who's Who In Frontier Science and Technology; Supervision of dissertation research of Rosemarie M. Booze, "Preferential vulnerability of the immature hippocampus to triethyl lead: A probe of behavioral and neural development," through the Johns Hopkins University; Invited participant to Symposium on Developmental Neurotoxicology at the Annual Meeting of the Western Pharmacology Society, California; Invited presentation at the Fourth International Conference on Toxicity of Selected Chemical Agents: Neurotoxicology of the Fetus and Child, Arkansas; Invited presentation at the Collaborative Behavioral Teratology Study Symposium, Cincinnati; Invited paper presented to the Department of Psychology, St. Lawrence University, New York; Invited paper presented to the Neurotoxicology Division of the Environmental Protection Agency, North Carolina; Ad Hoc Reviewer for Developmental Psychobiology, Neurobehavioral Toxicology and Teratology, Neurotoxicology, Obstetrics and Gynecology, Physiology and Behavior, and Physiological Psychology.

Dr. D. I. McRee: Adjunct Associate Professor, NCSU; Coordinator US-USSR Cooperative Program on Health Effects of Physical Environmental Factors; Organized and lead a seven person delegation to the Soviet Union for participation in a workshop and to develop a two year work plan; Hosted two Soviet delegations to the U.S.; Representative for DHHS on Interagency Advisory Committee on Electric Field Effects from High Voltage Transmission Lines; Member of American National Standards Institute C95 Committee on Safety Standards for Non-Ionizing Radiation; Appointed to National Research Council Committee on Biological Effects of Non-Ionizing Radiation; Appointed member of IEEE's Committee on Man and Radiation (COMAR); Elected to membership on USA Commission A (Electromagnetic Metrology) of the International Union Radio Science; Editorial Review Board of Environmental Health Perspectives; Reviewer of manuscripts for Science, The Institute of Electrical and Electronics Engineers, Radiation Research, Health Physics, and Bioelectromagnetics Journal; Invited reviewer of Dosimetric Handbook for Non-Ionizing Radiation Research for the Air Force; Invited reviewer of "Recommended Occupational Exposure Standard for Radiofrequency Radiation" for NIOSH; Project Officer on Contract with North Carolina State University. Invited seminars "Physiological Effects of Non-Ionizing Radiation", NCSU and "Biological Effects of Non-Ionizing Radiation", Duke University.

Dr. C. L. Mitchell: Adjunct Professor, Department of Pharmacology and the Neurobiology Program, University of North Carolina, lectures presented to medical graduate and undergraduate students of the University of North Carolina; Member, Editorial Board, Environmental Health Perspectives; Member, Editorial Board, Neurotoxicology; Member, Editorial Board, Neurobehavioral Toxicology and Teratology; Member, National Institute of Environmental Health Sciences Labor Management Committee; Chairman, Committee on Methods in Neurobehavioral Toxicology, International Programme on Chemical Safety, World Health Organization; Reviewer of manuscripts for Toxicology and Applied Pharmacology, and Journal of Medicinal Chemistry; Participant in a US-USSR Workshop in the Soviet Union.

Dr. Hugh A. Tilson: Adjunct Associate Professor, Department of Zoology, North Carolina State University; Adjunct Associate Professor, Toxicology Curriculum, University of North Carolina; Associate Editor, Neurotoxicology; Member, Editorial Board, Neurobehavioral Toxicology and Teratology; Member, Editorial Board, Toxicology and Applied Pharmacology; Invited speaker, Third International Conference on Neurotoxicology of Selected Chemicals: Pyrethroids and Neuroactive Pesticides, Little Rock, Arkansas, September, 1984; Invited participant, International Workshop on Experimental Models of Treatment Effects on Central Nervous System Function and Behaviour: A Multidisciplinary Analysis, Rome, Italy, September, 1984; Invited speaker, Symposium on the Use of Organochlorine Insecticides in Neurobiology, ASPET, August, 1985; Program Chairman and Co-organizer of Fourth International Conference on Neurotoxicology of Selected Chemicals: Neurotoxicology in the Fetus and Child, Little Rock, AR, September, 1985; Invited participant, Symposium and Workshop, Design Considerations in Screening for Behavioral Teratogens, Cincinnati, Ohio, September, 1985; Invited panelist, Animal Models of MPTP Toxicology at Symposium on MPTP: A Parkinsonian Syndrome Producing Neurotoxin, Bethesda, MD, June, 1985; Invited seminar, "Neurochemical and Neurobehavioral Effects of DDT," Division of Neurotoxicology, Environmental Protection Agency, May, 1985; Invited seminar, "Neurobehavioral Techniques in Toxicology," Ciba-Geigy, Basel Switzerland, October, 1984; Invited seminar, "Neurochemical Correlates of DDT-induced Neurotoxicity," Medical College of Virginia, Department of Pharmacology, Richmond, VA, February, 1985; Invited seminar, "Experiments that Attempt to Correlate Neurochemical and Neurobehavioral Effects: Problems and Examples," Department of Psychology, North Carolina State University, Raleigh, NC, March, 1985; Invited seminar, "Neurobehavioral Toxicology, A Systematic Approach to the Study of the Nervous system," Division of Toxicology, Hoffman-LaRoche, Nutley, NJ, April, 1985; Consultant, NCTR Collaborative Study on Behavioral Teratology; Ad Hoc Expert Consultant, WHO International Program on Chemical Safety (IPCS), Acrylamide and Dimethyl Sulfate, Carshalton, England, December, 1984; Ad Hoc Expert Consultant, WHO IPCS, Committee to Draft Document on Neurotoxicology, Prague, Czechoslovakia, September, 1984; Lectures to graduate and medical students at North Carolina State University and Duke Medical School; Ad Hoc reviewer of Cooperative Agreements and Program for Environmental Protection Agency; Member, Animal Care Committee, NIEHS; Ad Hoc Reviewer for Brain Research, Psychopharmacology, Pharmacology, Biochemistry and Behavior, Journal of Pharmacology and Experimental Therapeutics, Fundamental and Applied Pharmacology and Teratology.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 50005-11 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Effects of Noise and Ototoxic Agents on Energy Balance and Metabolism in Cochlea		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Teruzo Konishi	Medical Officer (Research) LBNT NIEHS
Others:	J. Muratsuka	Visiting Fellow LBNT NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Neurophysiology		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.1	OTHER: 0.4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The perilymphatic perfusion with artificial perilymph containing sodium bromate (1mM) was employed in guinea pigs to study the ototoxicity of bromate. Changes in the <u>cochlear potentials</u> and <u>electrolyte concentrations</u> in the endolymph were measured in both control and experimental perfusions. The perilymphatic application of bromate resulted in a gradual suppression of the endocochlear potential and sound evoked responses. The recovery of the cochlear potentials was not observed. A substantial decrease in potassium and chloride concentrations and increase in sodium concentration were observed in the endolymph. Our results indicated that the primary site of action of bromate was on the stria vascularis and in advanced stage of intoxication bromate might affect the hair cells, causing a further suppression of the hair cell responses.		

PROJECT DESCRIPTION

METHODS EMPLOYED: Clinical reports indicated that bromate poisoning caused irreversible sensorineural hearing loss as well as kidney failure. Preliminary animal experiments attempting to examine the action of sodium bromate indicated that the systemic administration of bromate resulted in suppression of cochlear microphonics and endocochlear potential. Since the systemic administration of bromate causes severe nephrotoxicity and deteriorating general conditions, the precise mechanisms underlying the ototoxicity of bromate are still obscure. As bromate is stable and is not metabolizable in the body, the local application of bromate into the guinea pig cochlea was used to examine the ototoxic action of bromate. Guinea pigs were anesthetized with combination of ketamine and pentobarbital sodium. The endocochlear potential and K^+ or Cl^- activity in the endolymph were continuously measured with double-barreled ion selective electrodes. The cochlear microphonics and compound action potential were also recorded with differential electrodes placed in the basal turn of the cochlea. The perilymphatic space was perfused with artificial perilymph containing 1 mM sodium bromate. The rate of perfusion was 2 μ l/min and its duration was 60 min. Samples of the endolymph and perilymph were collected 60 min after the end of perfusion. The concentrations of K^+ and Na^+ were measured with a helium glow photometer and Cl^- concentration was determined using the microtitration technique developed by Ramsay et al. The osmolarity was also measured employing the freezing point depression.

MAJOR FINDINGS AND PROPOSED COURSE:

1. Cochlear potentials.

In control experiments in which the perilymphatic space was perfused with artificial perilymph the endocochlear potential showed a tendency to increase during perfusion and its mean increase was 5.9% at the end of perfusion. During the 60 min period after perfusion the endocochlear potential gradually returned to the preperfusion level. At the end of perfusion the normalized cochlear microphonics and compound action potential was 86.1% and 110.9% respectively.

When the perilymphatic space was perfused with artificial perilymph containing 1 mM of sodium bromate, the endocochlear potential showed an initial increase followed by a gradual decrease. The mean loss of the endocochlear potential was 12.6% at the end of perfusion. The endocochlear potential continued to decrease and no recovery was observed after the perfusion. The mean loss was 50.6% 60 min after the end of perfusion. The cochlear microphonics and compound action potential decreased gradually during perfusion and their losses were 12.6% and 50.7% respectively at the end of perfusion.

2. Electrolyte concentrations and osmolarity in the endolymph.

The K^+ and Cl^- activities in the endolymph measured with ion selective electrodes were 112.8 ± 2.06 mEq/l and 99.2 ± 0.9 mEq/l respectively. In control perfusion no substantial changes in K^+ or Cl^- activities in the endolymph were observed during and after perilymphatic perfusion.

During the perilymphatic perfusion with bromate solution the K^+ activity in the endolymph increased slightly and the mean increase was 6.1 mEq/l at the end of perfusion. Thereafter the K^+ activity in the endolymph decreased continuously and its loss was $10.5 \pm 4.2 \text{ mEq/l}$ 60 min after the end of perfusion. The increase of Cl^- activity in the endolymph was $4.6 \pm \text{mEq/l}$ at the end of perfusion. The loss of the Cl^- activity measured 60 min after the end of perfusion was $7.4 \pm 1.9 \text{ mEq/l}$.

The K^+ , Na^+ and Cl^- concentrations in the endolymph sampled 60 min after perfusion with the control solution were $157.8 \pm 4.3 \text{ mM}$, $0.4 \pm 0.1 \text{ mM}$ and $142.2 \pm 1.3 \text{ mM}$ respectively which showed no significant changes as compared to the endolymph electrolyte concentrations in nonperfused cochlea. The electrolyte concentrations in the endolymph showed marked decreases in perfusion with bromate. The endolymph K^+ concentration was $144.0 \pm 4.5 \text{ mM}$, Na^+ concentration was $2.6 \pm 0.5 \text{ mM}$ and Cl^- concentration was $126.2 \pm 1.2 \text{ mM}$. The osmolarity of the endolymph decreased to $282.7 \pm 3.8 \text{ mOsm/kg}$.

The project has been terminated due to the untimely death of Dr. Konishi.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The specific ototoxicity of bromate will be revealed by the results obtained by these studies together with the morphological studies which are in progress. The present study will make a significant contribution for understanding the ototoxicity of bromate. This project will be a significant part of the overall Institute program which provides an essential knowledge base on the impact of environmental factors on human health.

PUBLICATIONS

Konishi, T. and Mori, H.: Permeability to sodium ions of the endolymph-perilymph barrier. Hearing Research 15: 143-149, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50015-11 LBNT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of Microwaves on Neural Response

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Donald I. McRee	Research Physicist	LBNT	NIEHS
Others:	Clifford L. Mitchell	Supv. Pharmacologist	LBNT	NIEHS
	Luke Lee	Visiting Fellow	LBNT	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Non-Ionizing Radiation

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

0.6

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Frog sciatic nerves have been exposed to continuous wave (CW) and pulse microwave radiation. Rate of fatigue or loss of vitality (the ability of the nerve to continue firing under rapid stimulation) was increased in the nerve exposed to 2.45 GHz at a specific absorption rate of 10 mW/g. In order to determine if sine-wave modulated microwaves had an increasing effect on ionic transport as reported in the literature to occur in chick brain, frog sciatic nerves were exposed to 2.45-GHz microwaves sine-wave modulated at 8, 16, and 32 Hz. It was found that a 50 mW/g specific absorption rate was required to obtain a loss in vitality with this form of radiation. This result suggests that the nerve vitality is nonlinear with respect to microwave intensity. This type of nonlinear behavior would be expected if the neural membrane is acting as a diode-like detector of the microwave field. Additional nerves were exposed 2.45 GHz (CW) microwaves at SARs of 4, 7, and 10 mW/g at a temperature of 24°C. The increase in rate of fatigue was significant at 7 and 10 mW/g but not at 4 mW/g. The effect occurred after approximately 20 minutes of exposure at 10 mW/g and 1 hour at 7 mW/g. Nerves were exposed to 10 mW/g at ambient temperatures of 20° and 28°C. The refractory period was shortened at 28°C, and the latency was increased at 20°C.

PROJECT DESCRIPTION

METHODS EMPLOYED: Isolated neurons such as the sciatic nerve of the frog, lobster ganglia, and aplysia abdominal ganglia will be exposed to CW, pulsed and modulated microwave radiation at levels which produce different electric field intensities inside the tissue. The interaction of these electric fields with nerve function including membrane gating and ionic transport will be investigated. These results could provide a better understanding of the function of excitable membranes.

MAJOR FINDINGS AND PROPOSED COURSE: We have determined that microwave radiation does affect isolated sciatic nerves of the frog. This effect was seen as a loss in vitality when the nerves were rapidly stimulated. Use of various modes of delivery of microwaves (continuous wave, pulse wave, and frequency modulated waves) indicate that the effect is nonlinear involving rectification across the membrane. This type of nonlinear behavior would be expected if the neural membrane is acting as a diode-like detector of the microwave field.

In order to investigate the mechanism for the loss in vitality of frog sciatic nerves exposed to CW microwaves, nerves were treated with ouabain before being exposed. In this case no difference in loss of vitality was observed between the exposed and nonexposed nerves. Since ouabain blocks the activity of the $\text{Na}^+ - \text{K}^+$ pump, this result suggests that the microwave effect on nerve vitality are associated with the decay of ionic gradients normally maintained by active transport.

In experiments to determine if temperature in conjunction with microwaves altered the firing patterns of frog sciatic nerves, isolated nerves were exposed to 2.45 GHz continuous wave microwave radiation at 20° , 24° , and 28°C . The specific absorption rate was 10 mW/g. An increased rate of fatigue was found at 20°C and 24°C . Nerves exposed to microwaves at 20°C had a shorter latency (time from stimulus to peak of action potential). Nerves exposed at 28°C had a shorter refractory period (time following stimulation to reach original state).

Further studies will be aimed at determination of the interaction mechanisms producing the effect and to gain basic information on membrane function (gating, transport, permeability). The lobster giant axon will be used in these studies. If the lobster nerve is affected in the same way as the frog sciatic nerve, experiments will be carried out to explain why the change is occurring. It is believed that the loss in vitality of the nerve is due to an interaction with the long-term regulatory process - such as the maintenance of adequate ionic concentration gradient across the membrane - rather than an interference with the action potential firing mechanism. Therefore, our initial approach will be directed at effects on ionic transport through the membrane and ionic depletion. $\text{Na}^+ \text{K}^+ \text{ATPase}$ activity will be measured in exposed and control nerves. Membrane channel blockers and radioactively labelled ions (uptake and perfusion technique) will be used to evaluate these mechanisms.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROGRAM OF THE INSTITUTE: This research will contribute to the base of knowledge on the physiology of excitable membranes and will provide information on how membrane function is altered when perturbed by an environmental agent - in this case, microwave radiation. This research is

directed toward the mission of the Institute to conduct biomedical research concerned with the effects of environmental agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50038-07 LBNT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of 2450 MHz Microwave Radiation on the Cardiovascular System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Donald I. McRee Research Physicist LBNT NIEHS

Others: Clifford L. Mitchell Supervisory Pharmacologist LBNT NIEHS
Michael J. Galvin Physiologist RGP-EP NIEHS

COOPERATING UNITS (if any)

Duke University

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Non-Ionizing Radiation

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.4

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objectives of this project are to determine the influence of microwave radiation on cardiac tissue using in vitro and in vivo methodologies. A method for exposing isolated rat atria to microwave radiation has been developed. The data suggest that 2.45 GHz CW microwave radiation of 2 or 10 mW/g has no overt effect on the rate of force of contraction of isolated atria. In addition, the response of atria to drugs was not influenced by microwave exposure. Specifically the dose response curve for isoproterenol and acetylcholine was not influenced by either 1, 10 or 100 mW/g exposure and the ability of propranolol and atropine to inhibit the isoproterenol and acetylcholine response of rat atria was not altered by these exposure levels. Also, certain biochemical and physiological parameters, which are indicative of cardiac integrity, have been measured in unanesthetized rats during whole body ventral exposure to 2450 MHz CW microwaves. The data suggest microwave exposure of 10 mW/cm² for 6 hr has no effect on mean arterial blood pressure of colonic temperature. However, there was a microwave induced bradycardia which was exhibited after 30 min of microwave exposure at 10 mW/cm² and persisted throughout the remainder of the 6 hr exposure period. None of the biochemical or hematologic indices examined were influenced by this exposure level. Rats were also exposed to 2450 MHz CW microwaves for 6 hrs at 10 mW/cm² in an ambient temperature of 27°C. No change in heart rate was observed. When rats were exposed to 2450 MHz CW microwaves for 6 hrs at 10 mW/cm² in an ambient temperature of 30°C, tachycardia was measured.

PROJECT DESCRIPTION

METHODS EMPLOYED:

- a. Isolated rat atria maintained at either 22⁰ or 37⁰C were exposed to microwave radiation at specific absorption rates of 1, 2, 10 and 100 mW/g and rate and contractile force were monitored. For each experiment 2 pairs of atria were used, one control and one exposed, which were placed in specially designed tubes located in a waveguide exposure apparatus. In addition, the response of the tissue to sympathetic and parasympathetic agents has been determined during microwave exposure. The drugs used included propranolol, isoproterenol, acetylcholine and atropine.
- b. Adult male rats were exposed to whole body microwave radiation of 2 and 10 mW/cm² at carefully controlled temperatures and exposure levels. Using a specially designed irradiation chamber, rats were exposed ventrally, and certain hemodynamic (blood pressure, heart rate), hematologic and biochemical parameters were measured during 6 hr microwave exposure. The temperature, humidity and noise level in the exposure chamber were maintained at either 23⁰, 27⁰ or 30⁰C, 60% and 70 dB respectively during the experimental period.

MAJOR FINDINGS AND PROPOSED COURSE:

- a. The data indicate that the exposure rates used (2 and 10 mW/g) have no overt effect on the rate or force of contraction of isolated atria at either incubation temperature. At 22⁰C the rate of contraction was 102 beats per minute for both the control and exposed atria. Atria maintained at 37⁰C had a rate of contraction of 215 beats per minute, and was also unchanged by microwave exposure. These experiments have been extended to examine the response of atria to drugs during microwave exposure. Dose response curves to various sympathetic and parasympathetic acting agents were determined for atria exposed to 1, 10, or 100 mW/g. Microwave exposure had no influence on the atrial response to the tested agents. In addition, the action of sympathetic and parasympathetic antagonists on exposed atria were not altered by microwave exposure.
- b. The data from these experiments indicate that ventral microwave radiation of rats (10 mW/cm²) at 22⁰C, induced a bradycardic after 1 hr of exposure. Specifically, in the sham exposed rats the mean arterial blood pressure (MABP), heart rate (HR) and colonic temperature were 120 + 10 mmHg, 300 + 30 BPM, and 37.5 + 0.8⁰C respectively. Six hour exposure to 10 mW/cm² microwave radiation had no influence on MABP or temperature. However, there was a highly significant reduction in heart rate which was evident within 60 min after the beginning of the exposure and persisted for the remainder of the experiment. In addition, a number of hematologic and biochemical parameters were examined during the exposure period. No differences between the sham (0 mW/cm²) or exposed (10 mW/cm²) groups were noted for any of these parameters. Rats were also exposed to 2450 MHz for six hours at 10 mW/cm² in an ambient temperature of 27⁰C. No change in heart rate was observed. When rats were exposed to 2450 MHz CW microwaves for 6 hr at 10 mW/cm² in an ambient temperature of 30⁰C,

tachycardia was measured. Since microwave irradiation does not effect the pacemaker cells of the heart and since the effect on the heart rate is a function of both ambient temperature and microwaves, we believe that the effect on the heart rate is due to effects on the autonomic nervous system as the animals try to maintain homeostasis.

This project has been completed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The potential health effects of microwave radiation in the environment is of interest to NIEHS. This project is designed to provide a comprehensive and integrated study of the possible effects of microwave radiation on the cardiovascular system. By using in vivo techniques microwave interactions with cardiovascular system can be evaluated more effectively. This research on the effects of microwaves on the cardiovascular system is directed toward the mission of NIEHS to determine the health effects of physical factors in the environment.

PUBLICATIONS

McRee, D.I., Galvin, M.J., and Mitchell, C.L.: Microwave effects on the cardiovascular system: A model for studying the responsivity of the autonomic nervous system to microwaves. Electromagnetic Waves and Neurobehavioral Function, Alan R. Liss, Inc., New York, 1985, in press.

Galvin, M.J., Jeffreys, L.M., and McRee, D.I.: Influence of microwave radiation on the response of spontaneously beating rat atria to sympathetic and parasympathetic agents. Bioelectromagnetics, 1985, in press.

Galvin, M.J. and McRee, D.I.: Cardiovascular, hematologic, and biochemical effects of acute (6 hr) ventral exposure of conscious rats to 2450 MHz (CW) microwave radiation. Bioelectromagnetics, 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 50076-04 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Effects of Noise and Drugs on Water Control of the Cochlear Fluids		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Teruzo Konishi	Medical Officer (Research) LBNT NIEHS
Others:	Y. Muratsuka H. Ueda	Visiting Fellow LBNT NIEHS Guest Worker LBNT NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Neurophysiology		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 1.7	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The purpose of this study is to elucidate the mechanisms of water movement across the cochlear partition. Systemic injection of <u>glycerin</u> or <u>deionized water</u> was used to induce alterations in the <u>osmolarity</u> of the blood serum in guinea pigs. The <u>cochlear potential</u> including the <u>endocochlear potential</u> did not show marked suppression when the osmolarity of the cochlear fluids was elevated or lowered. Under normal conditions the osmolarity is significantly higher in the endolymph than perilymph. When the blood osmolarity was altered, the osmotic gradient across the cochlear partition decreased.</p> <p>These studies are used as base line data to which water control in the cochlear fluids under pathologic conditions can be compared.</p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: The cochlear fluids are in the state of dynamic balance with continuous exchange of ions and metabolites between the cochlear fluids and their environment. It has been speculated that ototoxic agents may lead to local or systemic disturbance in the ion and water exchange mechanisms. No quantitative changes in the osmotic pressure of the cochlear fluids or hydrostatic pressure gradient across the cochlear partition following administration of various ototoxic drugs or chemicals have been reported. The purpose of this study was to investigate the osmotic relation between the cochlear fluids, blood and cerebrospinal fluid (CSF).

Guinea pigs were anesthetized with combination of ketamine and pentobarbital sodium. The cochlear microphonics (CM) and compound action potential (AP) were recorded with differential electrodes placed in the basal turn of the cochlea. The double-barreled ion selective microelectrodes were used to record the endocochlear potential (EP) and ionic activity in the endolymph continuously. Similar ion selective electrodes were utilized to record ion activity in the perilymph. Samples of the cochlear endolymph and perilymph were also collected. The CSF was collected by puncture of the atlantooccipital membrane. Osmolarity of the cochlear fluids and CSF was determined using the freezing point depression. Osmolarity of blood serum was determined with a vapor osmometer. The K^+ and Na^+ concentration was measured with a helium glow photometer and the Cl^- concentration was determined by the microtitration method.

Osmolarity of the blood was altered by intravenous injection of 3 g/kg glycerin or intraperitoneal injection of 1 ml/10 g BW of deionized water.

MAJOR FINDINGS AND PROPOSED COURSE:

1. Nontreated guinea pigs.

The osmolarities of the perilymph in the scala vestibuli and the scala tympani were 290.1 ± 5.2 mOsm/kg and 290.3 ± 4.4 mOsm/kg respectively. The osmolarity of the endolymph was 301.5 ± 5.3 mOsm/kg which was significantly higher than perilymph. The osmolarity of CSF was 289.1 ± 3.0 mOsm/kg and the blood osmolarity was 290.2 ± 5.9 mOsm/kg.

2. Guinea pigs with hyperosmolarity induced by glycerin injection.

a) Cochlear potentials.

The EP measured prior to glycerin injection was 85.1 ± 41 mv. It decreased to 80.2 ± 4.9 mv 10 min after injection of glycerin. Thereafter the EP gradually recovered and returned to the preinjection level. The EP measured 90 min after injection was 84.2 ± 5.8 mv. The sound evoked responses were also temporarily suppressed shortly after injection of glycerin. An increase of the positive summing potential was always accompanied with a decrease of CM. The CM gradually decreased and loss of CM was 10% 120 min after injection.

b) Ion concentration of the cochlear fluids.

The K^+ concentration in the endolymph increased continuously from 145.8 ± 11.8 mM to 156.9 ± 11.8 mM during the 60 min period after glycerin injection. During this period the K^+ concentration in the perilymph of the scala tympani also increased from 2.9 ± 1.1 mM to 3.8 ± 1.0 mM. The temporal changes in the Cl^- concentration in both endolymph and perilymph were found to be similar to those observed in K^+ concentrations of the endolymph and perilymph.

c) Osmolarity

The osmolarity of the blood serum rapidly increased and reached 343.6 ± 4.9 mOsm/kg 15 min after injection of glycerin. Thereafter the osmolarity of the blood serum decreased gradually and was 308.5 ± 7.2 mOsm/kg 3 hours after injection. The CSF showed the maximum osmolarity (329.0 ± 5.7 mOsm/kg) 60 min after injection. The osmolarity of the endolymph and perilymph increased continuously and 90 min after injection the osmolarity was 340.1 ± 16.6 mOsm/kg in the endolymph and 334.3 ± 15.7 mOsm/kg in the perilymph.

3. Guinea pigs with hypoosmolarity induced by water injection.

a) Cochlear potentials.

No marked changes in the EP was observed. The EP measured 60 min after intraperitoneal injection of water was 89.6 ± 4.5 mV which was comparable to the preinjection EP (89.4 ± 4.6 mV). The CM gradually decreased and reached 87% of the initial CM 60 min after injection.

b) Ion concentration of the cochlear fluids.

The K^+ concentrations in the endolymph and perilymph began to decrease 20 to 30 min after water injection and continued to decrease during the 60 min observation. The losses of K^+ and Cl^- concentration in the endolymph were 12.8 mM and 4.7 mM respectively. The K^+ concentration gradient between the endolymph and perilymph decreased as a function of time elapsed.

c) Osmolarity.

The osmolarity of the blood serum sampled 7 min after water injection showed loss of 13.6 mOsm/kg. Sixty min after injection its osmolarity decreased to 260 ± 15.4 mOsm/kg. No recovery was observed during 120 min after injection. The osmolarities of the endolymph and perilymph decreased to 276.3 ± 9.6 mOsm/kg and 273.2 ± 10.0 mOsm/kg respectively 2 hours after water injection.

These findings suggest that the endolymph-perilymph barrier is highly permeable to water and alteration of the blood serum osmolarity tends to decrease the osmotic gradient across the cochlear partition.

This project has been terminated due to the untimely death of Dr. Konishi.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The mechanisms involved in the kinetics of water in the cochlear fluids have been a subject of extensive studies but convincing data are still limited. These studies are issued as base line data to which water control in the cochlear fluids under pathologic conditions can be compared. This project will make a significant contribution to the Institute program which provides an essential knowledge base on the impact of environmental factors on human health.

PUBLICATIONS

Konishi, T., Hamrick, P. E., and Mori, H.: Water permeability of the endolymph-perilymph barrier in the guinea pig cochlea. Hear. Res. 15: 51-58, 1984.

Ueda, H., Muratsuka, Y., and Konishi, T.: Effect of glycerol on inner ear fluid electrolytes and osmolalities of guinea pigs. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 90030-05 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Effects of Toxicants on Membrane-Related Neurochemistry		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Stephen C. Bondy	Research Chemist LBNT NIEHS
Others:	H. Komulainen	Visiting Fellow LBNT NIEHS
	T. Walsh	Staff Fellow LBNT NIEHS
	H. Tilson	Pharmacologist LBNT NIEHS
COOPERATING UNITS (if any) U.S. Environmental Protection Agency		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Neurochemistry		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>One purpose of membrane-related studies has been to delineate injury to discrete neuronal tracts. This has the goal of identification of damage to specific circuitry which is especially vulnerable to a given neurotoxic agent. This project has involved study of the effect of various neurotoxic agents upon neurotransmitter translocations in the synaptic region. High-affinity uptake systems, calcium stimulated neurotransmitter release and assay of receptor binding sites have been carried out in animals treated with organometals. The differential susceptibility of various brain regions to trimethyltin and triethyl tin has been studied in this manner and data related to morphological findings. In the case of triethyl lead studies, biochemical behavioral correlations between analgesia and benzodiazepine binding sites have been made.</p> <p>A second goal is to study less selective damage to cerebral membranes. Such general effects may still present as specifically damaging certain nerve pathways, perhaps because of their intrinsic sensitivity to insult. This work focuses on the effect of agents upon levels of free calcium within the synaptosome and on depolarization-induced calcium fluxes across the synaptosomal membrane. Novel methods of assaying these parameters have been adapted for our studies. This work will also involve determination of membrane fluidity by use of fluorescent probes.</p>		

PROJECT DESCRIPTION

OBJECTIVES:

1. To develop the capacity to assay levels of ionic calcium within synaptosomes using a fluorescent dye and a continuous recording system.
2. To study the ability of various agents to modulate resting levels of synaptosomal calcium and rates of depolarization-induced calcium entry. Fluorescent data will be compared to parallel experiments assaying ⁴⁵Ca entry rates by rapid filtration techniques. Agents that will be studied include biogenic polyamines and chlordecone, which are suspected to modulate cerebral calcium metabolism.
3. To inquire whether alterations in intracellular calcium levels can be correlated with changes in the viscosity of the plasma membrane. Membrane fluidity will be assayed by use of fluorescent probes.

METHODS EMPLOYED:

Methods include the assay of ⁴⁵Ca uptake and release by synaptosomal preparations under various states of K⁺ induced depolarization. Incubation is terminated by rapid filtration and washing in order to remove non-accumulated calcium. Intracellular free calcium is assayed by markers (QUIN 2 or FURA) which have intensely fluorescent calcium complexes. These compounds are formed intrasynaptosomally after enzymic hydrolysis of the corresponding acetoxymethyl esters. This method allows a continuous dynamic recording of intrasynaptosomal events. Membrane fluidity is measured by assay of the rotation of polarized light passing through a synaptosomal suspension incubated with diphenylhexatriene (DPH). This lipophilic compound is inserted into the plasma membrane and the retardation of its free rotation is related to membrane viscosity.

MAJOR FINDINGS AND PROPOSED COURSE:Intracellular calcium levels

Major work has involved selection and development of an appropriate fluorescent compound with which to study low levels of calcium. The following conclusions have been made:

1. The compound FURA, first described in 1985, is superior to QUIN 2 (available since 1983 and relatively widely used), since
 - (a) It is more stable to the intense light used during fluorimetry.
 - (b) It is more sensitive than QUIN 2 so that less can be used. This has the advantages of minimizing any buffering effect on calcium levels and minimizing the formation of toxic products such as formaldehyde, during hydrolysis.
 - (c) It is effective precisely in the range needed for these studies 100-4000 nM calcium.

- (d) It allows direct rather than indirect measurement of calcium, by its wave-length-shift property.
 - (e) It is insensitive to zinc and magnesium, the latter being present in cells to a much greater extent than free calcium.
2. Preliminary studies with methyl mercuric chloride and chlordecone suggest that μM amounts of these compounds can elevate synaptosomal calcium levels. This work will also involve study of synaptosomes prepared from dosed rats. Putrescine which has been reported to mobilize intracellular calcium does not increase synaptosomal calcium following incubation at 10 μM putrescine.

Calcium uptake

The uptake of ^{45}Ca by synaptosomes is increased by over three fold in the presence of depolarizing amounts of potassium. While chlordecone does not alter the depolarization-induced component of ^{45}Ca entry, the resting level of ^{45}Ca is significantly elevated by chlordecone but not by DDT. In view of the differing responses of the tremor induced by these two organochlorines to phenytoin, this may be relevant.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Most neurotoxic agents also cause changes in organs other than the brain. This suggests that specific neuronal damage may be accounted for in terms of their especial susceptibility to changes that may be rather broadly based. A leading candidate for the ubiquitous factor underlying many forms of cell injury are elevated levels of intracellular calcium. Such increases are potentially lethal to cells. However, long before such major changes, neurons may express altered characteristics due to relatively minor changes in their content of free calcium. This is because of the special role that calcium plays in facilitating neurotransmission. Furthermore, other specialized neuronal features such as axoplasmic transport, are also calcium-dependent.

This research may reveal changes in neuronal membrane viscosity and rates of calcium uptake, that precede the neurotransmission alterations penultimate to behavioral expression.

PUBLICATIONS

Werdel, J.M. and Bondy, S.C.: Uptake of blood-borne materials by the chick retina and brain during visual deprivation. Brain Res. 301: 259-263, 1984.

Hong, J.S., Hung, C.R., Seth, P.K., Mason, G., and Bondy, S.C.: The results of manganese treatment on the levels on neurotransmitters, hormones, and neuropeptides: Interaction of stress with such effects. Environ. Res. 34: 242-249, 1984.

Ali, S.F., Abou-Donia, M.B., and Bondy, S.C.: Modulation of avian muscarinic high-affinity binding sites by a neurotoxic organophosphate. Neurochem. Pathol. 2: 267-275, 1984.

Bondy, S.C. and Uphouse, L.L.: Neurobiology and behavior - a critical interface. Interdisciplin. Sci. Rev. 9: 358-364, 1984.

Bondy, S.C. and Hall, D.L.: Effect of acute triethyl lead treatment on metallo-enzymes and binding characteristics of rat brain hippocampus. Neurochem. Pathol. 2: 251-266, 1984.

Bondy, S.C.: Chlorinated compounds and chlordecone. In Manzo, L. and Blum, K. (Eds.): Neurotoxicology. New York, Marcel Dekker, 1985.

Bondy, S.C., Walsh, T.J., Hong, J.S., Hall, D.L., and Tilson, H.A.: Effects of triethyl lead on hot plate responsiveness and biochemical changes in the hippocampus. Pharmacol., Biochem., and Behavior (In Press).

Bondy, S.C.: The biochemical evaluation of neurotoxic damage. Fundamental and Applied Toxicology (In Press).

Bondy, S.C.: Biochemical contributions to neurotoxicology. In Galli, C.L., Manzo, L., and Spencer, P.S. (Eds.): Toxicology of the Nervous System. Plenum Press, 1985, (In Press).

Bondy, S.C.: Especial consideration for neurotoxicological research. CRC Reviews in Toxicology (In Press).

Bondy, S.C.: The neurotoxicity of organic and inorganic lead compounds. In Bondy, S.C. and Prasad, K.N. (Eds.): Neurotoxic Metals. Karger Press, (In Press).

Veronsi, B. and Bondy, S.C.: Triethyltin-induced damage in neonatally exposed rats. Neurotoxicol. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 90031-04 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Assessment of Neurophysiological Effects of Organometals		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> PI: C. L. Mitchell Supv. Pharmacologist LBNT NIEHS </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Membrane Physiology		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS: <div style="text-align: center;">1</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.5</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The organometals have numerous applications in industrial and occupational settings. The neurotoxicity of these agents, particularly <u>organoleads</u> and <u>tins</u>, is well known. However, their precise sites and mechanisms of action are poorly understood. The purpose of these studies is to characterize the neurophysiological effects of relevant organometals in an attempt to determine the <u>site</u> of action and aid in determining the <u>mechanism</u> of action of selected organometals. We have found that triethyl lead (TEL), but not trimethyl lead, triethyl tin or trimethyl tin markedly increases the sensitivity to pentylenetetrazol induced seizures. This increase in sensitivity is most pronounced in animals receiving multiple pentylenetetrazol injections. There is little, if any, change in sensitivity to a single dose of pentylenetetrazol. Thus, the most striking effect of TEL appears to be an acceleration of the pentylenetetrazol kindling process. This effect of TEL can be attenuated by anticholinergic agents (atropine and scopolamine). Assessment of the neurophysiological and neurochemical bases for pentylenetetrazol-induced kindling and the mechanisms whereby TEL exacerbates it should further our knowledge of excitatory phenomena of the nervous system and how these can be altered by neurotoxic substances. </p>		

PROJECT DESCRIPTION

OBJECTIVES: Exposure to many organometals results in a pattern of toxicity resembling that produced by lesions of the limbic forebrain. However, the precise sites and mechanisms of action are poorly understood. Moreover, little is known concerning the structure activity relationships of these chemicals.

Our objective is to characterize the neurophysiological effects of organolead and organotin compounds in an attempt to determine anatomical sites of action and aid in determining their mechanisms of action. The working hypothesis is that for triethyl lead and trimethyl tin, at least, the limbic system will be perturbed by concentrations of these agents which do not affect other areas of the nervous system.

METHODS EMPLOYED: The initial approach was to determine the single dose, 28 day LD50 by the subcutaneous (SC) route for trimethyl lead (TML), triethyl lead (TEL), trimethyl tin (TMT), and triethyl tin (TET) in Fischer 344 rats. This was done in order to determine a common basis for the selection of doses of those agents in future studies. Subsequently, the ability of 1/4, 1/2, or 3/4 of the LD50 for each of the compounds to exacerbate convulsive activity elicited by pentylenetetrazol (PTZ) was examined. Times after toxicant administration most commonly studied were 1, 7, 14, 21, and 28 days. The purpose of these studies was to establish the doses and time intervals to be utilized for subsequent electrophysiological and neurochemical investigations.

The next step is to investigate the integrity of the limbic system by after-discharge, kindling, and evoked potential techniques utilizing the dosages and time intervals determined from the preceding studies. Comparisons are then made with other systems (e.g., auditory, visual somatosensory) to ascertain the relative specificity of any effect observed. In addition, the ability of anticholinergic and gabanergic agents to alter the effects of the organometals are also investigated.

MAJOR FINDINGS AND PROPOSED COURSE: The effects of TEL, TML, TET and TMT on PTZ-induced seizures have been investigated. PTZ was administered interperitoneally (I.P.) in a dose of 35 mg/kg. This dose reliably produced a mild array of epileptiform signs in the normal Fischer 344 rat. After the animal was treated with PTZ, it was immediately placed in a sound-attenuating chamber and observed for a period of 10 minutes. Any epileptiform signs and their latency were recorded. Two designs have been used to date. The first is a repeated measures design wherein the rats are treated with a range of dosages of any organometal or with the saline vehicle, and then are tested at 1, 7, 14, 21 and 28 days after this acute treatment. This design allows for the assessment of seizure susceptibility and the development of kindling among the rats. The second design is a non-repeated measures one. In these studies the animals are given injections of the metals or saline at 28, 21, 14, 7, or 1 day prior to the single PTZ challenge. Thus, each animal is treated only once with the PTZ and the possible confound of kindling development is controlled.

TEL, but not TML, TMT or TET markedly increases the sensitivity to PTZ. This increase in sensitivity is most pronounced in animals receiving multiple PTZ

injections. There is little, if any, change in sensitivity to a single dose of PTZ. Thus, the most striking effect of TEL appears to be an acceleration of the PTZ kindling process. Moreover, anticholinergic agents (atropine and scopolamine) can attenuate this effect of TEL.

Since TEL produced the most striking effect, subsequent studies will concentrate on this compound, using the others where appropriate to determine the specificity of the TEL effects. The following are examples of possible approaches:

1. The neurotoxicity of TEL, TML, TET and TMT on the limbic system will be assessed by investigating their effects on the propensity to (a) lower the threshold for electrically induced afterdischarge in various limbic structures, and (b) to alter the characteristics of evoked potentials.
2. Comparison of intralimbic system effects of microinjection of kainic acid or AF64A (an agent which selectively lesions cholinergic neurons) with similar injections of the organometals using the electrophysiological procedures mentioned above.
3. Use of the in vitro hippocampal slice technique to compare single or multiple unit potentials from selected portions of the hippocampus in treated and control animals. This can be useful both for localizing effects of the organometals within the hippocampus and studying mechanisms. The latter can be done by adding neuropeptides, neurotransmitters or trace metals to the bath to study how these alter the unit potentials.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Organometals are found with great frequency in the environment, yet there is little known about the site(s) and mechanism(s) by which these agents produce their neurotoxicity. The results of this research will provide significant data on where within the brain and how these agents affect neural tissue. This information will be useful in the development and assessment of logical strategies for the detection of toxicity and/or treatment of exposed individuals. In addition it should increase our understanding of the epileptic kindling process and how TEL exacerbates it.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 90033-03 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Milk Bombesin		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Lawrence H. Lazarus Research Chemist	LBNT NIEHS
Others:	W. E. Wilson Research Chemist	LBNT NIEHS
	B. J. Irons Biological Technician	LBNT NIEHS
	A. Guglietta Visiting Fellow	LBNT NIEHS
COOPERATING UNITS (if any) University of Turin, Italy University of North Carolina, Chapel Hill Kyoto University, Japan University of Rome, Italy		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Peptide Neurochemistry Group		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 1.5	OTHER: 1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) A peptide containing immunoreactivity to the amphibian peptide bombesin was isolated from bovine milk. The isolation procedure, however, gave relatively poor yields in spite of the precautions taken against loss due to absorptivity on the walls of various containers. New procedures are being developed to increase the yield of peptide recovered and decrease the number of steps in the scheme. Amphibian and mammalian bombesin-like peptides are known to affect gastric secretions either by i.p. infusion of the peptide or by intracerebral ventricular administration; a bombesin-like peptide in milk may have a similar mode of action. Thus, a preliminary assessment was under taken on a large number of potential analogues of milk bombesin on their neuromodulatory role of gastric secretion in rats. The data can be summarized as follows: (i) Trp in position 8 is required for biological activity; (ii) Gln and His at positions 6 and 12, respectively, are also important; and (iii) unknown extension of sequences preceeding Gln ₆ are involved since neuro-medin C (GRP 18-27) appears inactive.		

PROJECT DESCRIPTION

METHODS EMPLOYED: The bombesin-like peptide was purified from 1.2 kg. lots of powdered bovine milk as follows: (a) acid solubilization; (b) ammonium sulfate precipitation; (c) gel-filtration chromatography; (d) preparative ODS LC chromatography using step-wise elution with acetonitrile; (e) semipreparative ODS LC chromatography employing linear acetonitrile gradients and (f) analytical HPLC with various reverse-phase supports in combination with gradient and isocratic elution using acetonitrile and propanol. Gastric secretion was measured by intracerebral ventricular administration of peptides in rats previously fitted with cannulae. The contents of the stomachs were measured for volume, pH, hydrogen ion concentration and output.

MAJOR FINDINGS AND PROPOSED COURSE: The isolation scheme developed resulted in a purified peptide, however, with very low yields. As a consequence of these observations, we inoculated rabbits with a bombesin antigen in order to obtain a large quantity of antiserum: the IgG fraction was isolated and an immunoaffinity column prepared. The introduction of this procedure is crucial to the isolation of large amounts of peptide in relatively high yields required for sequence studies. A synthetic peptide will enable us to conduct physiological and pharmacological studies in vivo.

The intracerebral ventricular administration of bombesin-like peptides induced changes in gastric secretion. Those findings that specific amino acid residues of bombesin are required to elicit an effect on gastric secretion will enable us to further characterize the milk peptide. The chemical synthesis of analogues of milk bombesin by Prof. H. Yajima (Kyoto University) will help to define the specific amino acid sequences required for bioactivity. Furthermore, it will also permit us to develop specific antagonists against a mammalian bombesin-like peptide. Monoclonal antibodies will be produced in order to procure a highly specific reagent for studies on the tissue distribution of the milk bombesin molecule and characterize the role of the peptide in milk.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM AT THE INSTITUTE: The isolation of a new peptide related to the amphibian peptide bombesin will lead to increased knowledge on the neuromodulation of gastric secretion. Bombesin-like peptides apparently directly regulate gastric acid and gastrin in the stomach which may be involved in exacerbating the symptoms of ulcers. The development of analogues will aid in the recognition of a bombesin peptidergic pathway affecting several neurologically mediated responses; e.g., gastric secretion, satiety, thermoregulation, dispogenicity, and gastrointestinal hormone release. Perturbation of a well-established neuroregulatory mode by environmental factors may prove to be useful as tools to further examine the role of bombesin on various physiological parameters, behavior and growth promotion.

PUBLICATIONS

Lazarus, L.H., Wilson, W.E., Gaudino, G., Irons, B.J., and Guglietta, A.: Evolutionary relationship between nonmammalian and mammalian peptides. Peptides, in press, 1985.

Guglietta, A., Strunk, C.L., Irons, B.J., and Lazarus, L.H.: Central neuromodulation of gastric secretion by bombesin-like peptides. Peptides, in press, 1985.

Gaudino, G., Fasolo, A., Merlo, G., Lazarus, L.H., Renda, T., D'Este, L., Melchiorri, P, and Vandesande, F.: Active peptides from amphibian skin are also amphibian neuropeptides. Peptides, in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01-ES-90034-02 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Rabbit Stomach Peptide [Physalaemin-like Material (PLIM)] in Mammalian Tissue		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	William E. Wilson	Research Chemist LBNT NIEHS
Others:	L. H. Lazarus B. H. Irons A. Guglietta C. Hamm D. Harvan	Research Chemist Biological Technician Visiting Fellow Electronics Technician Chemist (formerly with LBM, NIEHS)
COOPERATING UNITS (if any) University of Kyoto, Kyoto, Japan University of Rome, Rome, Italy University of North Carolina, Chapel Hill, N.C.		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Peptide Neurochemistry		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 1.5	OTHER: 1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> By utilizing an antiserum which recognized the amino-terminal region of the amphibian peptide, physalaemin, it has been possible to isolate several cross-reacting peptides from rabbit stomach; these peptides have been designated physalaemin-like immunoreactive peptides (PHLIPs) PHLIP-8 has the following structure: <Glu-Val-Asp-Gln-Ala; PHILIP-7 is identical to PHLIP-8 in the amino acid sequences 1-7. These peptides do not possess tachykinin properties characteristic of amphibian physalaemin or of Substance P; they appear to be degradation products of larger molecules which have recently been recovered from rabbit stomach tissue. Because several of the biological properties of the PHLIP precursors are similar to those of physalaemin, efforts are underway to purify these larger peptides. It is possible that the PHLIPs are ultimately derived from stomach glycoproteins, e.g., mucin. Since material which cross-reacts with physalaemin antiserum has been detected in central nervous system tissue, it is probable that such material is structurally more similar to the PHLIP precursors than to physalaemin; antisera will be obtained for PHLIP-8 and will be used to redetermine the content and distribution in the central nervous system of PHLIP cross-reacting materials. </p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: Physalaemin-like immunoreactive peptides (PHLIPs) have been purified using several schemes. The initial steps in purification have involved ethanol-formic acid extractions of lyophilized rabbit stomachs, followed by removal of ethanol and concentration of the neutralized extracts. Molecular sieving and ion-exchange chromatography have been utilized as initial steps in purifying the extracts. HPLC techniques were then used to facilitate final purifications. Peptide sequence elucidation was facilitated by utilization of fast atom bombardment-tandem mass spectrometry. Amino acid analyses were performed using HPLC techniques. Peptides were synthesized by manual solid phase techniques. Biological activities involved recording contraction characteristics of smooth muscle preparations (such as guinea pig ileum and rabbit large intestine) and monitoring blood pressure in vivo.

MAJOR FINDING AND PROPOSED COURSE: Two PHLIPs were purified from rabbit stomach. The structure of PHLIP-7 is <Glu-Val-Asp-Pro-Asn-Ile-Gln; PHLIP-8 is identical in positions 1-7 and contains Ala as the C-terminal amino acid; structures were confirmed with synthetic peptides.

Smooth muscle contractility characteristics of physalaemin are not mimicked by PHLIP-8 when comparable concentrations of peptides are tested; however, PHLIP-8 has been demonstrated to influence smooth muscle contractility when exposure has involved micromolar, rather than nanomolar, quantities of peptide. The lack of tachykinin-like properties of PHLIP-8 was anticipated from its structure.

Preliminary efforts to recover the PHLIP precursor(s) have indicated that those molecules may be very large; in fact, the ultimate precursor may be a large glycoprotein such as a mucin. Current efforts are underway to devise techniques to purify the precursor molecules.

The proposed course is to obtain antisera to PHLIP-8. The antisera will permit formation of immunoaffinity columns to facilitate future purification efforts; it will also permit reexamination of previous results which indicated that similar molecules exist in central nervous system tissue, particularly, in the lower brain and spinal cord areas.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The 80% structural homology in pentapeptide amino-terminal sequences of physalaemin and the PHLIPs confirms an evolutionarily conserved portion of epithelial secretory products. Since this sequence has been found in respiratory, nerve (including spinal cord), and gastrointestinal tissues, it may be involved in multiple physiological roles. If, as we suspect, the PHLIPs are fragments of glycoproteins, further investigations of structure and distribution may lead to a better understanding of disorders involving glycoproteins such as mucous in the gastrointestinal tract and lungs (eg. cystic fibrosis). In the central nervous system, knowledge of structures and distribution of the PHLIP precursors may provide insight into any of a number of disease states involving altered glycoprotein metabolism.

PUBLICATIONS

Wilson, W.E., Harvan, D.J., Hamm, C., Lazarus, L.H., Klapper, D.G., Yajuma, H.: Physalaemin-like peptides from rabbit stomach: purification and elucidation of structure using fast atom bombardment-mass spectrometry. 9th American Peptide Symposium, Toronto, Ontario, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 90035-02 LBNT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of Ornithine Decarboxylase in the Detection of Tissue Activation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Stephen C. Bondy	Research Chemist	LBNT	NIEHS
Others:	J. S. Hong	Pharmacologist	LBNT	NIEHS
	C. L. Mitchell	Pharmacologist	LBNT	NIEHS
	H. A. Tilson	Pharmacologist	LBNT	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Neurochemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to account for the rapid changes in levels of ornithine decarboxylase (ODC) in tissues that respond to damage by regenerative or adaptive changes. The role of ODC is somewhat understood in relation to cell division but the function of this enzyme in non-proliferating tissues is unknown. Chlordecone administration to rats, at levels causing tremor (40 mg/kg body weight) causes a 21-24-fold increase in levels of adrenal ODC. This is a rapidly occurring, reversible event of much greater magnitude than any other biochemical response to chlordecone hitherto reported. The onset of chlordecone induced tremor can be prevented by pretreatment with an irreversible inhibitor of ODC, difluoromethylornithine (DFMO). This implies a relation between polyamines and behavioral responses. However, cerebral ODC levels are not markedly elevated in chlordecone-treated rats. Cerebral ODC is dramatically elevated following electroconvulsive shock or after intracerebral injections of colchicine or kainic acid. The regional specificity and time course of these effects are currently under study.

The goals of this project are twofold:

1. Further understanding of the role of polyamines in the adult nervous system, especially in relation to neurotransmission.
2. The exploitation of the ODC assay in order to delineate the precise areas of the brain that may be initial targets of neurotoxic agents.

PROJECT DESCRIPTION

METHODS EMPLOYED: The main technique employed is the assay of ODC activity by trapping of $^{14}\text{CO}_2$ evolved during the decarboxylation of labeled ornithine. The instability of this enzyme and possibility of non-specific decarboxylation have to be taken into account.

OBJECTIVES: Three main objectives exist:

1. The detection of the primary site of action of organochlorines known to disrupt the hypothalamic-adrenal axis and definition of the components necessary for the expression of neurotoxicity.
2. The demonstration of the potential applicability of ornithine decarboxylase as a marker for abrupt neuroendocrine changes in response to environmental or chemical factors. The precise locus of commencement of a series of adaptive modifications may be located in this manner.
3. Further understanding of the role of polyamines in the regulation of neuronal activity.

MAJOR FINDINGS AND PROPOSED COURSE: ODC is an enzyme involved in polyamine synthesis. Elevated levels of this enzyme imply high levels of cellular activity. Such activity may include proliferation, hypertrophy, secretion, growth and regeneration. The short half life (20 min) of this enzyme allows rapid detection of the onset of any of the above processes.

1. Chlordecone causes an increase in adrenal ODC levels that is both rapid (within 2 hr) and persistent. This increase is also found in the adrenals of hypophysectomized rats and may reflect the activation of adrenal medulla as well as cortex. It is not clear whether chlordecone directly affects the adrenal gland or whether all effects are mediated by hypothalamic secretion of ACTH. This question is being further addressed by study of the effects of chlordecone upon cortical and medullary cells in culture and by the use of spantichotomized animals where the adrenal medulla is atrophied.
2. The tremor induced by chlordecone treatments of rats is blocked by a prior treatment with dimethylfluorornithine (DFMO), an irreversible inhibitor of ODC. This inhibition can be reversed by concurrent administration of putrescine, the product formed by ODC. Radioactive DFMO has been shown to penetrate the brain. This suggests a facilitative effect of polyamines upon chemically induced tremor. Thus while DFMO has no behavioral effects when systemically administered, polyamines may modulate the adaptive responses of the brain to chemical insults.

Other organochlorine tremorigens or convulsants are currently under study, including DDT and lindane.

3. The calcium-stimulated release of d-aspartic acid following its accumulation into synaptosomes, is significantly inhibited in the presence of DFMO while

the simultaneous presence of putrescine reverses this effect. This implies that polyamines are involved in the regulation of neurotransmission. Currently the effect of polyamines (putrescine, spermine, spermidine) upon synaptosomal, depolarization-induced calcium uptake, is being studied.

4. The elevation of ODC in brain, after electroconvulsive shock (ECS) is regionally selective. Cerebellum and hippocampus are most responsive regions while ODC levels of striatum and hypothalamus are unaltered by this treatment. The precise time course of these events is under study. While fresh ODC synthesis can lead to elevated levels of this enzyme in 2-4 hours, unmasking of pre-existing inhibited enzyme may occur within minutes of ECS.
5. A new form of particulate ODC has been characterized in the brain. The authenticity of this enzyme has been established by pharmacological studies and demonstration of putrescine formation. This enzyme differs from its soluble counterpart in its pH optimum, Lineweaver-Burke kinetics, and response to ECS.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This work will throw light on potential areas which are those first affected by organochlorines and may allow identification of many known effects as indirect. A distinction between tremorigenic and non-tremorigenic organochlorines may lead to definition of more than one type of primary impact site.

The work also illustrates the potential of ODC as an index of tissue activity and thus a means of detection of focal sites of toxic damage. This enzyme, much exploited in cancer research, has great, largely unexplored value in studies of damage to nerve tissue.

The putative role of polyamines in the regulation of neurotransmission is a relatively new concept which has been hitherto little studied. Evidence suggests the putrescine may function as a second messenger and modulate intracellular calcium levels in a manner analogous to the recently described inositol triphosphate system.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 90036-02 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Animal Model of Organometal Neurotoxicity		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Hugh A. Tilson	Pharmacologist LBNT NIEHS
Others:	T. J. Walsh	Staff Fellow LBNT NIEHS
	J. S. Hong	Pharmacologist LBNT NIEHS
	R. L. McLamb	Technician LBNT NIEHS
	S. C. Bondy	Research Chemist LBNT NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Neurobehavioral		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Alkylmetals were studied for their potential to produce selective neurobehavioral and neurochemical alterations in limbic forebrain function. Short-term repeated or acute exposure to triethyl lead (TEL) impaired passive avoidance and facilitated two-way avoidance, effects similar to those observed in animals with limbic fore-brain lesions. However, subsequent work indicated that TEL did not affect working memory as determined by performance in the radial-arm maze. Comparisons of tri-methyl lead (TML) with TEL indicated that they produce quantitatively different behavioral effects, possibly due to toxicokinetic factors leading to differential neuromorphological changes in the CNS. Neurochemical studies indicated that TEL, TML and triethyl tin (TET) had few specific effects, but trimethyl tin (TMT) produced a pattern of neurochemical changes indicative of hyperammoniaemia. Although it is evident that TEL is not a limbic toxicant, other experiments demonstrated that it did produce specific alterations in neurobehavioral function (antinociception, increased responsiveness to pharmacological challenge) related to corresponding changes in neurochemical function (receptor binding, increased enzyme activity). Experiments with other potential limbic toxicants such as intracerebral administration of AF64A, a cholinergic cytotoxicant, produced signs of limbic forebrain dysfunction which were associated with decreases in acetylcholine (ACh) content in the hippocampus and frontal cortex. Destruction of granule cells in the dentate gyrus with colchicine produced similar behavioral effects. These studies indicate that cytotoxicants such as AF64A and colchicine may be useful in the study of neurode-generative diseases involving limbic forebrain function.		

PROJECT DESCRIPTION

RESEARCH PROJECT

1. Nature of the Problem: Clinical accounts of accidental human poisoning and toxicological studies in laboratory animals have demonstrated that the organometals, and in particular the trialkyl metals, are potent neurotoxicants. Several lines of investigation have provided compelling evidence that the limbic system is a highly vulnerable site for some organometal compounds. Little is known about the reason for the apparent vulnerability of the limbic forebrain to certain chemicals or the ability of the organism to compensate to toxic insult in this area.
2. Objectives: The purpose of the research is to: (a) address the hypothesis that the limbic system is a particularly vulnerable site for the expression of organometal compounds (OMs) by determining the time- and dose-related effects of alkylmetals, such as trimethyl tin and lead, on various aspects of neurobehavioral and neurochemical function and by comparing the behavioral effects of systemically administered alkylmetals with those produced by injecting cytotoxicants directly into limbic forebrain sites; (b) study the neurological basis for selected neurotoxic effects, such as antinociception, produced by triethyl lead (TEL); (c) initiate studies to develop strategies for the treatment of animals exposed to cytotoxicants.
3. Recent Accomplishments and Significance: Initial studies determined the neurobehavioral effects of short-term repeated exposure to TEL chloride. Responses indicative of limbic system damage (i.e., facilitated two-way avoidance, impaired passive avoidance) and altered sensorimotor function (increased motor activity, enhanced startle response) were seen in TEL-exposed rats. An unusual finding was that TEL appeared to decrease responsiveness to a mild thermal stimulus (antinociception). Subsequent experiments sought to determine systematically the effects of TEL and related OMs following acute administration. Triethyl tin (TET), trimethyl tin (TMT) and trimethyl lead (TML) were all found to decrease responsiveness to a thermal stimulus, but each had a different time course following acute administration. Neurochemical studies comparing the four OMs found little consistent effect on neurotransmitters and neuropeptides in various regions of the CNS. However, TMT decreased putative inhibitory transmitters glycine, taurine and GABA in the hippocampus and frontal cortex; these effects were correlated with onset and duration of behavioral effects. Region wide increases in glutamine suggested that TMT alters the disposition of glutamine and/or glutamate or that it increases ammonia content in the brain. TML, TET, and TEL had no effect on amino acid neurotransmitters. The effect of TEL on cognitive function as measured by performance in the radial arm maze was studied since this measure is believed to be a sensitive indicator of hippocampal function. TEL had no significant effect on rate of acquisition or performance following insertion of various delays between trials.

More intensive comparisons of the neurobehavioral effects of TEL and TML indicated that these agents produce quantitatively different effects on

sensorimotor function which appear to be due to toxicokinetic factors resulting in different exposure of critical areas in the nervous system. This interpretation is supported by the observation that the onset and duration of lead levels in the blood and brain following TEL or TML were different and that TEL produced structural abnormalities (cellular swelling) in the hippocampus and dorsal root ganglion (hypertrophy and accumulation of mitochondria), while TML-exposed rats had neuromorphological changes confined primarily to the spinal cord and brain stem.

In summary, these experiments indicate that in the adult rat neither TEL nor TML have specific effects on the structure or function of the limbic forebrain. However, other studies have indicated that when administered neonatally, TEL can produce specific damage to the CA3 region of the hippocampus. In addition, our experiments indicate that TMT, an agent reported by other laboratories to produce relatively specific damage in the limbic forebrain, had minimal selectivity for this brain site. One explanation for this finding is that the strain of rat used in our laboratory, the Fischer-344 albino, may be less sensitive to the limbicotoxic effects on TMT and other OMs relative to the strain of rat used by other laboratories.

The mechanism of TEL-induced antinociception was examined in a series of studies. Acute administration of TEL was found to be unrelated to the neuro-motor impairment and to have a topography unlike that of opiate analgetics such as morphine. It was subsequently found that manipulation of test variables such as familiarity with the apparatus and test environment could modify TEL's antinociception. For example, repeated exposure to a non-functional hot-plate as well as daily hot-plate testing attenuated the antinociception induced by TEL. These studies suggest that TEL-induced antinociception is related to an enhanced behavioral and/or biological response to stress and/or novelty. Previous studies indicated that chlordiazepoxide attenuates the effects of short-term repeated exposure to TEL, an observation supported by neurochemical studies showing that TEL-induced increases in hot-plate latencies are correlated with changes in flunitrazepam binding in the hippocampus. Other experiments have shown that pharmacological antagonism of benzodiazepine receptors with RO 15, 1788 also produced time- and dose-related antinociception. Recent pharmacological experiments have indicated that cholinergic and serotonergic antagonists had no effect on TEL-induced antinociception. Higher doses of naloxone attenuated the effects of TEL, and TEL was found to be additive with morphine, suggesting a role of endogenous opiates in TEL's effect. Pharmacological studies have indicated that dopamine receptor agonists enhance antinociception produced by TEL, while depletion of brain catecholamines decreased it. Acute exposure to TEL has also been found to enhance the motor activity increasing effects of d-amphetamine and apomorphine; TEL did not alter the distribution or time course of d-amphetamine, but did alter DA-stimulated adenylate cyclase and increase the number of DA receptors.

In summary, although TEL may not produce specific limbic forebrain damage, this agent does produce specific neurobehavioral effects associated with alterations in CNS structure and function.

As part of the current research program, various neurotoxicants, including organometal compounds, are being used to study the neurobiological role of the limbic forebrain. Initial studies found that injection of AF64A, a cholinergic cytotoxicant, directly into the lateral cerebroventricles increased motor activity, impaired passive avoidance retention, and impaired performance in the radial arm maze. One interpretation of these effects is that AF64A interrupted cholinergic input into the hippocampus which was verified by injecting AF64A directly into the dorsal hippocampus, corpus striatum or nucleus basalis. Destruction of granule cells in the dentate gyrus with colchicine produced similar behavioral deficits. In addition, rats exposed to colchicine were more sensitive to the effects of scopolamine, but not d-amphetamine, indicating an increased sensitivity of cerebral cholinergic receptors. Histological analysis revealed that colchicine destroyed most of the granule cells in both the superior and inferior blades of the dentate gyrus while sparing pyramidal cells in CA1, CA2, CA3, CA4 and GABAergic interneurons throughout the hippocampus. Additional experiments with DSP-4, a selective neurotoxicant for norepinephrine, indicated that depletion of brain catecholamines does not interfere with performance in the radial arm maze.

In summary, experiments with cytotoxicants such as AF64A and colchicine indicate that these agents may be useful tools to study function of specific pathways in the hippocampus and the control of cognitive function and motor activity.

PLANS FOR THE SUBSEQUENT YEAR:

The potential for the nervous system to recover following exposure to toxic insult or to demonstrate adaptive or compensatory responses following environmental change will be examined in future studies. Initial studies will examine the possible functional role of various membrane-bound neuronal components in neural regenerative processes. One such neuronal component are the gangliosides which are sialic acid-containing glycosphingolipids associated with most cells. The gangliosides are believed to play a distinctive role in neuronal differentiation and regeneration. Research will be initiated to determine the conditions and neural systems in which gangliosides have neuritogenic and neuronotrophic properties.

The purpose of the proposed research is to establish the neurochemical and neurobehavioral effects following exposure to specific cytotoxicants such as colchicine or 6-hydroxydopamine and determine the capacity of ganglioside exposure to prevent the effect or promote the recovery of behavioral function and to associate that recovery, if possible, to specific changes in neurochemical capacity. Depending upon the success of initial work with gangliosides, other agents such as phosphatidylserine, the interleukins or glycoproteins will be considered.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

This research seeks to determine the neurobiological effects of neurotoxicants such as the alkylmetals and cytotoxicants in an attempt to develop animal models of certain neurodegenerative diseases. This information will be useful in the development of strategies for treatment and prevention of neurodegenerative changes following exposure to toxic insult.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 90037-02 LBNT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Toxicological Perturbations of Behavioral and Neural Development		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Charles F. Mactutus	Senior Staff Fellow	LBNT NIEHS
Others: Hugh A. Tilson John S. Hong Rosemarie M. Booze Meta Bonner	Pharmacologist Pharmacologist Guest Worker Graduate Student	LBNT NIEHS LBNT NIEHS LBNT NIEHS LBNT NIEHS
COOPERATING UNITS (if any) The Johns Hopkins University Duke University		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Neurobehavioral		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 2	PROFESSIONAL: 1	OTHER: 1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Longitudinal and time-sequential studies have examined the dynamic interplay between the greater susceptibility to disruption and the greater capacity for reorganization and recovery (or sparing) of function of the immature nervous system. The functional outcome of these opposing processes was studied with respect to the model systems provided by the maturing hypothalamic-pituitary-adrenal axis and the hippocampus. Exposure to various toxicants was used to perturb these systems. Neonatal exposure to chlordecone, an organochlorine insecticide, produced imbalances in circulating and adrenal steroids as well as extremely rapid and apparently permanent changes in adrenal morphology. These alterations in the integrity of the hypothalamic-pituitary-adrenal axis were associated with long-term alterations in the sexual differentiation of hypothalamic nuclei, neurochemical alterations in catecholaminergic and serotonergic function, and selective memory deficits. Neonatal exposure to triethyl-lead, the active metabolite of leaded gasoline, produced a preferential and permanent destruction of hippocampal pyramidal cell fields as indicated by quantitative neuromorphometry. This insult resulted in a permanent increase in behavioral reactivity independent of sensory modality, early undernutrition, and early testing effects. Pharmacological probes and receptor binding techniques linked this enhanced reactivity to a permanent dysfunction in cholinergic, but not dopaminergic, connections with the hippocampus proper. Initial studies which employed prenatal exposure to carbon monoxide, with carboxyhemoglobin levels within the range experienced by cigarette smokers, suggested a disruption of the hippocampus as indexed by an impairment in the acquisition and retention of a two-way avoidance task in juvenile aged offspring. While a substantial attenuation of these deficits was noted with maturation to adulthood, with continued aging there was a marked exacerbation of learning and memory dysfunction.		

PROJECT DESCRIPTION

METHODS EMPLOYED: Procedures developed in this laboratory are used to determine dose- and age-response functions for alterations in behavioral and neural development. We have focused primarily on studying the vulnerability of the maturing hypothalamic-pituitary-adrenal axis and the hippocampus to disruption by environmentally relevant compounds: chlordecone (an organochlorine insecticide), triethyl lead (the active metabolite of leaded gasoline), and carbon monoxide (at levels within the range experience by cigarette smokers). Functional measures of motor development, reflex development, habituation, and learning/memory are utilized. Parametric manipulations and pharmacological agents are employed to probe the functional integrity of neural and endocrine systems. Neuroendocrine and neuromorphological studies are also used to assess more molecular aspects of selected behavioral and/or neural dysfunction and potential recovery.

MAJOR FINDINGS AND PROPOSED COURSE:

1) Early perturbations of the hypothalamic-pituitary-adrenal axis and the hippocampus were observed following exposure to chlordecone (an organochlorine insecticide), triethyl lead (the toxic metabolite of leaded gasoline), and carbon monoxide.

Chlordecone: A series of tests was designed specifically for assessment of the known clinical signs and symptoms of chlordecone neurotoxicity. Of primary concern was the development of tests which could be used throughout the lifespan of the animal, with particular emphasis placed on equating tasks and parameters for the limited response repertoire of young animals. As indicated in studies of the acute pharmacological effects of such exposure to adult animals, neonatal chlordecone produced marked hyperreactivity, whole body tremor, and a depression in growth rate when observations were taken during the preweaning period (Mactutus et al., 1984). A developmental time course for each effect has been obtained.

A somewhat more persistent alteration in memory processes was also produced by chlordecone (Mactutus and Tilson, 1984; Mactutus, unpublished observations); in each case the paradigms permitted assessment of specific "associative" effects independent of generalized deficits in performance attributable to other factors; e.g., differential sensitivity to footshock, hyperreactivity to the conditional stimuli, differences in motor activity. In confirmation of prior observations, the chlordecone-exposed animals also displayed significantly higher levels of circulating plasma corticosterone than controls following the retention test (Mactutus and Tilson, 1984). The overresponsiveness of the hypothalamic-pituitary-adrenal axis was hypothesized to contribute, and perhaps underlie, these perturbations of development.

Triethyl Lead: Following neonatal exposure to triethyl lead (TEL), preweaning assessments indicated that TEL produced an acute but transient biphasic dose related response which was independent of undernutrition. An initial period of excitability, indicated by the presence of tremor, occurred during the second week of life. This phase was followed by one of reduced activity and auditory startle measurements. Weanling brain weights were depressed by neonatal TEL-exposure and quantitative morphometric analyses revealed a specific loss of hippocampal regio inferior pyramidal cells without evidence for widespread neural damage.

Carbon Monoxide: Prenatal carbon monoxide exposure (15.6% HbCO), at levels within the range experienced by cigarette smokers (1 - 16% HbCO), produced a slight but nonsignificant depression in birth weight of exposed animals. However, no differences in initial growth of the dams, number of pups per litter, sex ratio, or mortality on day 1 was observed, nor was there any evidence of gross structural deformity. Evaluation of learning and memory processes for a two-way active avoidance task indicated a marked impairment in associative ability of the prenatal carbon monoxide exposed animals during the postweaning period. Multiple dependent measures, specific control groups, and a replicate study confirmed that this deficit was independent of nonassociative or motivational alterations.

2) While such early alterations are potentially difficult to dissociate from acute pharmacological effects, persistent alterations in the hypothalamic-pituitary-adrenal axis and the hippocampus were nevertheless indicated upon maturation of the animals to adulthood.

Chlordecone: Dysfunction of the hypothalamic-pituitary-adrenal axis persisted well into adulthood. In confirmation of preliminary observation, depressions in the basal levels of circulating and adrenal corticosteroids have been observed (Rosecrans et al., 1984; 1985) as has adrenal hyperplasia (Mactutus, unpublished observations). Initial studies of the long-term effects of early chlordecone exposure also indicate an impairment in performance of a complex avoidance task. However, is not yet known whether this deficit is most parsimoniously attributable to a learning deficit, memory deficit, or some other nonassociative factor (e.g., attentional deficit or alteration in motivation). Additional longitudinal evaluations (Mactutus et al., 1984) also discovered alterations in the sexual differentiation of hypothalamic nuclei involved in feeding and weight regulation, and neurochemical alterations in catecholaminergic and serotonergic function. There were no significant residual effects detectable in the direct assessment of tremor and hyperreactivity, however, the modulation of these effects by early stressful experiences again suggested the contribution of hormonal factors to the observed alterations.

Triethyl Lead: Time-sequential testing of the consequences of early exposure to TEL demonstrated that these animals displayed a hyperreactive response in a number of behavioral tasks, e.g., a lack of habituation to activity testing, immobility during spectral analysis of body movement, increased auditory startle responsiveness, and increased intertrial activity in an avoidance learning task. In each of these tasks, TEL effects were detected in the absence of, or were greater than, effects due to early undernutrition. Analyses of regional brain weights indicated that only the hippocampus was depressed in wet weight in both sexes. Quantitative morphometry further indicated the selective cell loss in hippocampal areas CA3a and CA3b of TEL-treated animals was of a permanent nature. An examination of the functional status of dopaminergic and cholinergic connections with the hippocampus proper indicated that the behavioral hyperreactivity was not modulated by dopaminergic systems, but was altered by cholinergic manipulations in a gender-specific and task-specific manner. In sum, neonatal TEL-exposure was found to preferentially and permanently affect behavioral processes (hyperreactivity) and hippocampal neuronal integrity.

Carbon Monoxide: Time sequential studies were conducted to examine the long-term consequences, if any, of prenatal carbon monoxide exposure on learning and memory processes. Upon maturation to adult, the prenatal carbon monoxide exposed animals acquired a two-way avoidance task as equally well as controls. Although a subtle impairment in retention was suggested 24 hours later, the magnitude of the deficit was quite small relative to that observed in juvenile aged animals. However, extending the duration of the retention interval to 28 days produced a much stronger retention deficit. An additional study was conducted with similarly exposed animals which were permitted to mature to one year of age prior to evaluation. The prenatal carbon monoxide exposed animals showed marked impairments in both the acquisition and 24-hour retention of the avoidance retention of the avoidance task.

3) Our proposed course of study includes the extension of these findings to address (a) the mechanisms underlying the persistence of, and recovery from, early impairment in behavioral and neural function, and (b) whether any recovery of function (and presumably neural reorganization) is of adaptative or maladaptive significance, particularly with continued aging.

Chlordecone: A major emphasis will focus on the adaptative significance of the early hormonal imbalances induced by chlordecone and their consequences for behavioral and neural development. Initial studies will determine whether the hypothalamic-pituitary-adrenal axis and the central nervous system are target sites for the distribution of chlordecone in neonatal animals. The influence of neonatal chlordecone on the functional integrity of the HPA axis will then be determined by testing adrenal (ACTH injection) and pituitary reserve (metyrapone) with radioimmunoassays assayed for corticosterone and ACTH. Quantitative morphometry will also be used to examine the extent of structural damage to the adrenal gland. The more basic question of the role of early hormonal imbalance in the long-term consequences for behavioral and neural development will be similarly assessed, but using exogenous administration of various adrenal steroids rather than a neurotoxicant having multiplicity of biological effects. Doses of the steroids will be based on the known physiological capabilities of the developing HPA particularly interested in how such early manipulations may compromise the plasticity of the adult CNS with respect to learning and memory processes and subsequent response to further neural insult.

Triethyl Lead: We will investigate the putative excitatory and inhibitory neural circuits suspected of being responsible for the hippocampal pyramidal cell damage with specific pharmacological agents. Our major goal is to determine whether, following protection of the pyramidal cells from neuronal excitation with gabergic agonists and glutaminergic antagonists, TEL has a cytotoxic effect. If the pyramidal cells can be protected from any direct TEL induced damage, then cell death must be attributable to the pharmacological overexcitation of this pathway. Additional studies will be performed in conjunction with collaborators R. Booze and J. Davis at Duke University, to examine potential neuronal rearrangements which may underlie the persistent functional hyperactivity.

Carbon monoxide: Future studies will examine the two potential hypothesis concerning the same systems implicated by the use of chlordecone and triethyl lead: the hippocampus and the hypothalamic-pituitary-adrenal axis. In conjunction with Dr. Lawrence Fechter at the Johns Hopkins University, we will examine the generality of long-term alterations in memory retrieval processes to other behavioral paradigms. Morphologic evaluation of the hippocampus and adrenal gland will be performed. Tests of the capacity of the animal to respond to stress will also be evaluated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This research will provide information concerning the mechanisms and adaptative significance of perturbations of development of the hippocampus and the hypothalamic-pituitary-adrenal axis. In addition, the use of specific environmental agents as neurobiological probes should also provide information defining their locus and mechanism of neural insult. This increased understanding will be useful in developing effective therapeutic strategies following early toxicant exposure.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 90038-02 LBNT
PERIOD COVERED October 1, 1985 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Animal Model of Organochlorine Neurotoxicity		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Hugh A. Tilson	Pharmacologist
		LBNT
		NIEHS
Others:	J. S. Hong	Pharmacologist
	D. Herr	Guest Worker
	J. Gallus	Technician
		LBNT
		NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology		
SECTION Neurobehavioral		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The neuropharmacological basis for organochlorine-induced tremor and hyperexcitability was studied in rats. Both chlordecone and p,p'-DDT increased the release of brain norepinephrine and serotonin, while having marginal effects on dopamine; p,p'-DDT, but not chlordecone, was found to increase tissue levels of excitatory amino acids such as aspartate and glutamate in the brain stem and spinal cord. Pharmacological experiments to determine the functional significance of these neurochemical changes showed that cholinergic and serotonergic receptor antagonists attenuated the tremor produced by chlordecone, but enhanced that produced by p,p'-DDT. Blockade of alpha-noradrenergic receptors attenuated tremor produced by both organochlorines. Previous studies showed that pretreatment with the anticonvulsant phenytoin attenuated the tremor produced by p,p'-DDT, but enhanced that produced by chlordecone. Recent work extended this observation to augmentation of acoustic startle response produced by p,p'-DDT and chlordecone. Permethrin, a Type I pyrethrin believed to have the same mechanism of action as p,p'-DDT, produced the same neurochemical effects as p,p'-DDT; pretreatment with phenytoin also attenuated the neurological effects of permethrin. Intraventricular administration of calcium prior to the administration of p,p'-DDT or chlordecone attenuated or enhanced the tremorigenic effects produced by these agents, respectively. These experiments demonstrate the neurological manifestations produced by many of the organochlorines, such as tremor and augmented startle responsiveness, are similar, suggesting that they may activate a final common pathway; however, the neuropharmacological basis for the effect may be different. </p>		

PROJECT DESCRIPTION

RESEARCH PROJECT:

1. Nature of the Problem: The organochlorine insecticides represent a diverse class of chemicals having different behavioral and neurological effects in mammals. Some, such as chlordecone and p,p'-DDT, produce behavioral hyper-excitability and tremor, while others, such as lindane and dieldrin are primarily convulsants. Because these agents appear to act directly on some aspect of pre or postsynaptic function (gating mechanism, disposition of calcium), they have been used in vitro to study neural function. However, the relationship between the effects in vitro and those seen in vivo has not been well established.
2. Objectives: This research project is designed to address four questions: 1.) Can organochlorine-induced tremor be associated with specific alterations occurring at the neurochemical or neurophysiological level and can the functional relationship between those changes be demonstrated? 2.) Given that such a functional significance can be determined for one organochlorine, can the relationship be demonstrated for others in the same class? 3.) Given that a functional relationship exists for tremor, is the relationship the same for other neurobehavioral effects? 4.) Can certain organochlorines be used as probes to understand basic processes of neurological functioning?
3. Recent Accomplishments and Significance: A method to quantify tremor-related changes in whole body movement using spectral analysis techniques has been developed. Previous work showed that chlordecone-induced tremor can be differentiated from that produced by pharmacological agents, such as harmine, oxotremorine, or apomorphine, or following exposure to some physical factor such as cold-elicited shivering. Neurochemical studies with chlordecone showed a high statistical correlation between the onset and duration of tremor and increases in 5-HIAA and MHPG throughout the brain; changes in regional levels of amino acid concentrations were not observed. These results indicate that chlordecone increases the release of 5-HT and NE, but not amino acid transmitters, nonspecifically. Subsequent experiments showed that pharmacological blockade of serotonergic, noradrenergic and cholinergic receptors attenuates the tremorigenic effects of chlordecone. These data suggest that NE, 5-HT and ACh release contribute to the expression of chlordecone-induced tremor. Additional studies have indicated that the cerebellum is not needed for chlordecone-induced tremor and that pharmacological agents believed to inhibit polysynaptic reflexes attenuate chlordecone-induced tremor, while those that enhance these reflexes exacerbate tremor.

Recent studies with p,p'-DDT also indicate a good correlation between the onset and duration of tremor and brain 5-HIAA and MHPG. However, p,p'-DDT increased concentrations of excitatory amino acids glutamate and aspartate primarily in the brain stem and spinal cord. Experiments are now underway to determine the generality of these findings to permethrin, a Type I pyrethroid believed to have a mechanism of action similar to that of p,p'-DDT. Experiments with the relatively nonneurotoxic isomer of p,p'-DDT, o,p'-DDT,

and mirex, which has little neurotoxicity in mammals, failed to find significant alterations in either behavioral or neurochemical function at the doses used.

Since the possible mechanism of action of p,p'-DDT and permethrin appears to be on the activation gates of sodium (they hold the gate open once it opens), other pharmacological agents were used to try to block their effect on the sodium channel. One such agent is phenytoin, which is believed to block repetitive firing of nerves by binding to the inactivation gates of sodium. Pretreatment of animals with phenytoin significantly decreased the tremor produced by p,p'-DDT and permethrin, but increased the tremor produced by chlordecone. These data suggest that chlordecone has a different mechanism of action than p,p'-DDT and permethrin. Other studies are now underway to determine the differential effect of calcium on the effects of chlordecone and p,p'-DDT.

Additional experiments have been performed to quantify the effects of chlordecone and related insecticides on behavioral hyperexcitability as measured by the acoustic startle response. Dose- and time-effect experiments have been performed and the data indicate that chlordecone, p,p'-DDT, lindane and permethrin all augment the startle response. Analysis of startle responding within sessions indicate that each agent increases initial responsiveness (i.e., produces sensitization) and attenuates or blocks subsequent habituation of the response to repeated presentations of the stimulus. Additional experiments have indicated that pretreatment of rats with phenytoin attenuates the effects of p,p'-DDT and permethrin on the acoustic startle reflex, while phenytoin exacerbates the effects of chlordecone and lindane. These data suggest that the mechanism responsible for tremorigenesis is similar to that for enhanced startle reflex.

PLANS FOR THE SUBSEQUENT YEAR:

Because previous experiments indicated that phenytoin pretreatment attenuated the tremor and behavioral hyperexcitability produced by p,p'-DDT, additional studies will be done to determine the possible pharmacokinetic interactions between phenytoin and p,p'-DDT and whether or not pretreatment with phenytoin will block neurochemical changes produced by p,p'-DDT and chlordecone.

Previous experiments have indicated that pharmacological probes were useful in studying the functional significance of the neurochemical effects of chlordecone. This approach will be applied to the study of chlordecone- and p,p'-DDT-induced behavioral hyperexcitability. Studies are now underway to determine the role that the noradrenergic and serotonergic systems may play in the augmentation of startle responding produced by chlordecone and p,p'-DDT.

Thus far, only organochlorine-induced tremor and augmentation of acoustic startle responding have been examined systematically. Experiments are now underway to examine the effects of chlordecone and p,p'-DDT on reflex modulation as measured by the prepulse inhibition paradigm. Studies on the effects of acute and repeated exposure to organochlorines on learning and memory process are also underway.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

This research studies the functional relationship between the neurobehavioral and neurochemical effects of organochlorines. Data derived from these experiments will provide a basis for the development of strategies to treat or prevent signs following exposure to these agents. Elucidation of the site and mechanism of organochlorine-induced neurobehavioral effects will be useful to the study of neurodegenerative diseases involving the brain stem and related areas.

PUBLICATIONS

Gerhart, J.M., Hong, J.S., and Tilson, H.A.: Studies on the mechanism of chlordecone-induced tremor in rats. Neurotoxicol. 6: 211-230, 1985.

Hong, J.S., Tilson, H.A., Uphouse, L.L., Gerhart, J.M., and Wilson, W.K.: Effects of chlordecone exposure on brain neurotransmitters: Possible involvement of the serotonin system in chlordecone-elicited tremor. Toxicol. Appl. Pharmacol. 73: 336-344, 1984.

Tilson, H.A. and Mitchell, C.L.: Neurobehavioral techniques to assess the effects of chemicals on the nervous system. Ann. Rev. Pharmacol. Toxicol. 24: 425-450, 1985.

Tilson, H.A., Hong, J.S., Gerhart, J.M., and Walsh, J.J.: Animal models in neurotoxicology: The neurobehavioral effects of chlordecone (Kepone). In Thompson, T. and Dews, P. (Eds.): Advances in Behavioral Pharmacology. Academic Press, in press, 1985.

Tilson, H.A., Hong, J.S., and Mactutus, C.F.: Effects of 5, 5-diphenylhydantoin (Dilantin) on neurobehavioral toxicity of organochlorine insecticides and permethrin. J. Pharmacol. Exp. Ther., in press, 1985.

Chen, P.H., Tilson, H.A., Marbury, G.D., Karoum, F., and Hong, J.S.: Effect of chlordecone (Kepone) on the rat brain concentration of 3-methoxy-4-hydroxyphenylglycol: Evidence for a possible involvement of the norepinephrine system in chlordecone-induced tremor. Toxicol. Appl. Pharmacol. 77: 1985.

Herr, D.W., Hong, J.S., and Tilson, H.A.: DDT-induced tremor in rats: Effects of pharmacological agents. Psychopharmacology, in press, 1985.

Hudson, P.M., Chen, P.H., Tilson, H.A., and Hong, J.S.: Effects of p,p'-DDT on rat brain concentrations of biogenic amine and amino acid neurotransmitters and their association with p,p'-DDT-induced tremor and hyperthermia. J. Neurochem., in press, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 90039-02 LBNT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Modulation of Brain Opioid Peptides by Neuroleptics and Electroconvulsive Shock

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. S. Hong	Pharmacologist	LBNT	NIEHS
Others:	T. Kanamatsu	Visiting Fellow	LBNT	NIEHS
	S. P. Sivan	Senior Staff Fellow	LBNT	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Neuropharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

2

PROFESSIONAL:

1

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The first objective of this project was to develop methods to measure the turnover of brain enkephalin. We tried to measure the level of mRNA coding for enkephalin precursor (preproenkephalin A) as an index for the rate of biosynthesis by cell free translation or blot hybridization using cDNA clone for preproenkephalin A. The second objective was to examine the molecular mechanism underlying the modulation of opioid peptides by haloperidol, electroconvulsive shock (ECS) and Kainic acid (KA). Repeated injections of haloperidol caused a two-fold increase in the striatal concentration of enkephalin. This increase was accompanied by a two-fold increase in the level of mRNA coding for the precursor of enkephalin. This suggests that haloperidol accelerates the turnover of enkephalin. Furthermore, this study demonstrates that long-term treatment with haloperidol affects the gene expression of the enkephalin system. This finding raises an important consideration that gene expression may be the ultimate site of action for antipsychotic drugs. Similar to haloperidol, repeated ECS also increased the brain concentration of enkephalin and level of mRNA coding for preproenkephalin A. This finding lends further credence that gene expression may be a common site of action for various psychiatric treatments. A single injection of DA caused recurrent seizure and produced a three-fold increase in enkephalin concentration in the hippocampus 72 h post-dose. This increase in peptide level was preceded by a large increase in the abundance of mRNA coding for preproenkephalin A. This study suggests that the hippocampal enkephalin-containing neurons are responsive to the seizure activity induced by ECS or KA. For future studies, we plan to use the newly developed cell free translation and blot hybridization methods to study the biosynthesis of enkephalin after haloperidol or ECS in greater detail. These studies should provide further information regarding the possible role of enkephalin in mediating the actions of haloperidol and ECS.

PROJECT DESCRIPTION

RESEARCH OBJECTIVESa.) Development of turnover methods for opioid peptides.

For the last few years, progress of studying the turnover of peptides was hampered due to lack of methods. Our previous studies have been limited to the measurement of steady state concentrations of the peptides by radio-immunoassay (RIA). Since the steady state concentration does not precisely reflect the dynamic changes of peptide-containing neurons, development of turnover methods for opioid peptides is crucial for further understanding of the regulation of peptides. The first objective was to develop methods for measuring the biosynthesis of enkephalin in the brain. It is generally believed that the level of mRNA coding for enkephalin can reflect the rate of biosynthesis of this peptide. We tried to set up an in vitro cell free translation method to measure the translating activity of mRNA coding for preproenkephalin A. Additionally, we tried to develop a blot hybridization method using cDNA clone coding for preproenkephalin A to measure the level of mRNA coding for this peptide.

b.) Modulation of brain opioid peptides by haloperidol and seizure activity.

We have been interested in studying the regulation of [Met⁵]-enkephalin (ME) by psychotropic agents which are known to affect certain neurotransmitter systems. We have demonstrated that long-term treatment with haloperidol causes a profound effect on brain ME levels. This line of study has provided evidence indicating an intimate relationship between dopamine (DA) and ME in certain brain regions which are important sites of action of haloperidol. We have also discovered that the metabolism of brain opioid peptides can change in response to seizure activity induced by electroconvulsive shock (ESC) or kainic acid (KA) treatment. The purpose of this project is to study the molecular mechanism underlying the changes of opioid peptides after these treatments.

METHODS EMPLOYEDa.) Development of cell free translation and blot hybridization for measuring the level of mRNA coding for preproenkephalin A.

The detailed procedures of these two methods are described in the publication section:

Cell free translation	Sabol et al., 1983.
Blot hybridization	Hong et al., 1984.

b.) Modulation of brain enkephalinergic neurons after repeated injections with haloperidol.

Adult rats received daily injections of either saline or haloperidol (1 mg/kg, s.c.) for 3 weeks and were killed 24 h after the last injection. Striatum,

hypothalamus and frontal cortex were dissected. The levels of ME were determined by RIA. The levels of mRNA coding for preproenkephalin A were measured by cell free translation and blot hybridization.

c.) Modulation of brain enkephalinergic and dynorphinergic neurons after repeated ECS.

Rats received daily ECS for 6-10 consecutive days and were killed 24 h after the last shock. The brain levels of ME and dynorphin and mRNA level of preproenkephalin A were measured as described above.

d.) Modulation of brain enkephalinergic neurons after intrastriatal injection of KA.

Rats received a single injection of KA intrastriatally (1µg/rat) and were killed at different times thereafter.

MAJOR FINDINGS AND PROPOSED COURSE

a.) Development of in vitro cell free translation and blot hybridization methods for measuring the level of mRNA coding for preproenkephalin A.

We succeeded in setting up these two methods which allow us to study the changes of enkephalin turnover after different treatments or manipulations. With these two methods, the future studies on the regulation of peptide can be extended from the measurement of steady state level to the estimation of dynamic changes of the peptide-containing neurons.

b.) Modulation of brain enkephalinergic neurons after repeated injections with haloperidol.

Several studies have been carried out in an attempt to determine whether the elevation of striatal ME content after haloperidol treatment was due to an increase in the synthesis or due to a decrease in the utilization of ME. The rate of decline of striatal ME content in haloperidol-treated rats was steeper than that of controls after intraventricular injection of cycloheximide. These results suggest that haloperidol accelerates the turnover of ME. This hypothesis was confirmed by our recent findings that the activity or level of mRNA coding for preproenkephalin A, determined by cell free translation or in vitro hybridization using cDNA clones, was increased after repeated injects of haloperidol.

In summary, this study demonstrates that long-term treatment with haloperidol affects the gene expression of the enkephalin system. This raises an important consideration that gene expression may be the ultimate site of action for antipsychotic drugs.

c.) Modulation of brain enkephalin and dynorphin after repeated ECS.

This study estimated the biosynthesis of ME by measuring the level of mRNA coding for the precursor of ME, preproenkephalin A, using both in vitro cell

free translation or blot hybridization. Effects of repeated ECS on the concentrations of dynorphin in various brain regions were also studied. Ten daily ECSs caused a two-fold increase in ME-LI and an 80% increase in the level of mRNA coding for preproenkephalin A in the hypothalamus. These observations suggest that repeated ECS increase the biosynthesis of hypothalamic ME. Ten daily ECS also increased dynorphin A (1-8)-like immunoreactivity (DN-LI) in hypothalamus (50%), caudate nucleus (30%), septum (30%) but no significant change was found in the frontal cortex or the neurointermediate lobe of the pituitary. The most prominent effect of repeated ECS on the dynorphin system was in the hippocampus. Unlike the other brain regions, a 70% decrease of DN-LI was found in the hippocampus after 10 daily ECS whereas a significant increase of ME-LI (40%) was observed. Furthermore, immunocytochemical studies revealed that an increase in ME-LI occurred in the perforant pathway and a decrease in DN-LI occurred in the mossy fiber system after 10 daily ECS. These studies suggest that alterations in enkephalin and dynorphin in the limbic system may contribute to the behavioral changes observed after repeated ECS exposure.

In summary, this study demonstrated that repeated ECS alters the biosynthesis of brain opioid peptides. This study plus the aforementioned haloperidol study strongly suggests that the gene expression may be an important site of action for some psychiatric treatments such as antipsychotics or ECS.

d.) Modulation of brain enkephalin after intrastriatal injection of KA.

A single striatal injection of KA caused recurrent seizure activities lasting 3-6 h. During this period, there was a 30-40% reduction in the level of native ME-LI in the hippocampus. The ME-LI returned to control value 12 h postdosing and then showed a large rebound 24 h after postdosing (200% of control values); there was a three-fold increase in ME-LI 72 h postdosing. The reduction in ME-LI during the convulsing period suggests an increase in the release of this peptide and the rebound in ME-LI in later phase may be due to an overproduction of ME. This possibility was supported by a sharp increase in the abundance of mRNA^{enk} during the phase of recurrent seizures. The level of mRNA^{enk} reached a plateau (4 fold increase) 6 h postdosing then declined linearly and returned to normal value 72 h after injection. The increase in the level of mRNA^{enk} suggests an increase in the biosynthetic rate of enkephalin-containing neurons triggered by the seizure activities.

PLANS FOR SUBSEQUENT YEAR:

Studies of biosynthesis of brain opioid peptides after repeated administration of haloperidol, ECS and KA.

After developing these two in vitro methods for the measurement of mRNA coding for preproenkephalin, we plan to study the biosynthesis of enkephalin after haloperidol, ECS and KA in greater detail. Time course studies and detailed brain regional changes of enkephalin biosynthesis after haloperidol, ECS and KA are planned. These studies should provide further information regarding the possible role of enkephalin in mediating the actions of haloperidol, ECS and KA. Since levels of

dynorphin are affected by ECS, development of either cell free translation or blot hybridization method to measure the mRNA level coding for the precursor dor dynorphin is planned.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Elucidation of the regulatory mechanisms of neuropeptides may help us to learn more about the interactions between neurotransmitters and neuropeptides and the possible function of brain peptides. This type of information may provide the basis for our future studies regarding the significance of change in neuropeptide systems after animals are exposed to environmental neurotoxicants.

PUBLICATIONS

Hong, J.S., Yoshikawa, K., and Lamartiniere, C.A.: Hormonal regulation of pituitary endorphin systems. In Hanin, I. (Ed.): Dynamics of Neurotransmitter Function. Raven Press, 1984, pp. 149-157.

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Hong, J.S., Yoshikawa, K., Kanamatsu, T., McGinty, J.F., Mitchell, C. L., and Sabol, S.L.: Repeated electroconvulsive shocks alter the biosynthesis of enkephalin and concentration of dynorphin in the rat brain. Neuropeptides 5: 557-560, 1985.

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Rosecrans, J.A., Hendry, J.S., and Hong, J.S.: Biphasic effects of chronic nicotine treatment on hypothalamic immunoreactive β -endorphin in the mouse. Pharmacol. Biochem. Behav. (In Press).

Nakata, Y., Chang, K.J., Mitchell, C.L., and Hong, J.S.: Repeated electroconvulsive shock down-regulates the opioid receptors in rat brain. Brain Res. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 90040-02 LBNT																
PERIOD COVERED October 1, 1984 to September 30, 1985																		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) On the Possible Mechanism of Chlordecone-Elicited Tremor																		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: J. S. Hong</td> <td style="width: 30%;">Pharmacologist</td> <td style="width: 20%;">LBNT</td> <td style="width: 20%;">NIEHS</td> </tr> <tr> <td>Others: P. Chen</td> <td>Expert</td> <td>LBNT</td> <td>NIEHS</td> </tr> <tr> <td>P. M. Hudson</td> <td>Biological Lab Technician</td> <td>LBNT</td> <td>NIEHS</td> </tr> <tr> <td>J. Obie</td> <td>Biological Lab Technician</td> <td>LBNT</td> <td>NIEHS</td> </tr> </table>			PI: J. S. Hong	Pharmacologist	LBNT	NIEHS	Others: P. Chen	Expert	LBNT	NIEHS	P. M. Hudson	Biological Lab Technician	LBNT	NIEHS	J. Obie	Biological Lab Technician	LBNT	NIEHS
PI: J. S. Hong	Pharmacologist	LBNT	NIEHS															
Others: P. Chen	Expert	LBNT	NIEHS															
P. M. Hudson	Biological Lab Technician	LBNT	NIEHS															
J. Obie	Biological Lab Technician	LBNT	NIEHS															
COOPERATING UNITS (if any)																		
LAB/BRANCH Laboratory of Behavioral and Neurological Toxicology																		
SECTION Neuropharmacology																		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709																		
TOTAL MAN-YEARS: 1	PROFESSIONAL: 1	OTHER: 1																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <ol style="list-style-type: none"> 1. <u>To evaluate the possible role of sodium channel in the neurotoxicity of the organochlorine insecticides in vivo in rats.</u> <p>p,p'-DDT and related agents act to hold the sodium channel open once opened and this effect is believed to be responsible for neurological effects of tremor and hyperexcitability <u>in vivo</u>. Tremor was almost completely blocked in rats pretreated with hydantoin, an anticonvulsant believed to block repetitive firing of nerves by interfacing with the inactivation gates of sodium. A similar antagonism was observed for permethrin, a Type I pyrethroid believed to have a mechanism equal to that of DDT. However, hydantoin increased the tremorigenic effects of chlordecone, an organochlorine whose mechanism has not been linked to the sodium channel. Our data are consistent with the hypothesis that <u>in vivo</u> neurotoxicity of some organochlorine insecticides is related to their effects on the sodium channel.</p> 2. <u>To characterize neurochemical effects of chlordecone on the hypothalamo-pituitary-adrenal axis.</u> <p>A single injection of a tremorigenic dose of chlordecone (75 mg/kg, i.p.) increased the levels of plasma and adrenal corticosterone and the plasma level of ACTH. The increase in the pituitary-adrenal activity is consistent with morphological observations which indicate that adrenal cortical cells and corticotrophs of the pituitary hypertrophy after chlordecone exposure. Pretreatment with phenoxybenzamine and pizotifen (a 5-HT receptor blocker) completely prevented chlordecone-elicited increase in plasma levels of ACTH and corticosterone suggesting that monoamine mechanisms within the hypothalamus may mediate the neuroendocrine effects of chlordecone on the pituitary-adrenal axis.</p> 																		

PROJECT DESCRIPTION

RESEARCH OBJECTIVES:

- a.) To evaluate the possible role of the sodium channel in the neurotoxicity of organochlorine insecticides in vivo in rats.

The purpose of this study was to test the possibility that the prolongation of sodium channel may be related to organochlorine-elicited tremor and hyperexcitability.

- b.) To characterize neurochemical effects of chlordecone on the hypothalamo-pituitary-adrenal axis.

The purpose of this study was to examine the effect of chlordecone on hypothalamo-pituitary-adrenal function by determining: 1) the rate of turnover of monoamines in the hypothalamus, 2) the levels of ACTH in the pituitary and plasma, 3) the levels of corticosterone in the adrenal gland and plasma. Morphological studies of the pituitary and the adrenal from chlordecone-treated rats were also performed at the light and electron microscope levels.

METHODS EMPLOYED: To assess the effects of organochlorine on brain neurotransmitter systems, rats received a single injection of tremorigenic dose of chlordecone (75 mg/kg, i.p.) or p,p'-DDT (75 mg/kg, P.O.). Tremor activity was monitored at different time periods post-dose with spectral analysis. After completion of the measurement of tremor activity, rats were killed either by decapitation or by microwave irradiation and brains were dissected according to the method of Glowinski and Iversen. Levels of amines and their metabolites were measured by either high performance liquid chromatography (HPLC), or gas chromatography-mass spectrometry (GCMS). Turnover rates of Ach and GABA were measured by GCMS. Levels of amino acid transmitters were measured by HPLC.

To evaluate the contribution of each neurotransmitter system in the tremorigenesis of chlordecone and p,p'-DDT, pharmacological challenge studies were performed. Rats received a single injection of chlordecone or p,p'-DDT. Five hr post-dose, various compounds (agonists or antagonists specific to a neurotransmitter receptor) were injected and tremor activities were measured 30 min afterward. Modifications of chlordecone-induced tremor activities by these pharmacological agents may suggest a possible involvement of a neurotransmitter system in the tremorigenesis of this insecticide.

To characterize the neurochemical effects of chlordecone on the HPA axis, levels of ACTH in the pituitary and plasma and levels of corticosterone in the adrenal and plasma were measured by RIA after chlordecone treatment. Morphological studies of the adrenal and pituitary glands were also performed at the light and electronmicroscope levels.

MAJOR FINDINGS AND PROPOSED COURSE:

1. Possible role of the sodium channel in the neurotoxicity of organochlorine insecticides in vivo in rats: p,p'-DDT and related agents act to hold the sodium channel open once opened and this effect is believed to be responsible for neurological effects of tremor and hyperexcitability in vivo. Our lab has found that DDT-induced tremor and hyperthermia are highly correlated with an increase in the levels of the metabolites of norepinephrine (NE), serotonin (5-HT) and, to a lesser extent, dopamine (DA) in the brain stem, hypothalamus (HYP), striatum (STR), or hippocampus (HPC). DDT also increases levels of excitatory amino acids glutamate (GLU) and aspartate (ASP), but the effect occurs only in the brain stem. These effects are dose- and time related. Pharmacological studies found that blockade of α_1 -adrenergic, 5HT-1, or ASP, but not DA, receptors attenuate DDT-induced tremor. This neurological effect was almost completely blocked in rats pretreated with hydantoin, an anticonvulsant believed to block repetitive firing of nerves by interfering with the inactivation gates of sodium. A similar antagonism was observed for permethrin, a Type I pyrethroid believed to have a mechanism similar to that of DDT. However, hydantoin increased the tremorigenic effects of chlordecone, an organochlorine whose mechanism has not been linked to the sodium channel. Our data are consistent with the hypothesis that the in vivo neurotoxicity of some organochlorine insecticides is related to their effects on the sodium channel.

2. Neurochemical effects of chlordecone on the hypothalamo-pituitary-adrenal axis.

A single injection of tremorigenic dose of chlordecone (75 mg/kg, i.p.) increased the levels of plasma and adrenal corticosterone (4-5 fold above control level) 2 h post-dose and this increase persisted up to 7 days. Following the same treatment the plasma level of ACTH was increased 4-5 fold 2 h post-dose and remained elevated up to 4 days but returned to control value 7 days after chlordecone injection. On the other hand, such treatment caused a biphasic change of the pituitary level of ACTH: An initial reduction (40% during 2-24 h), then return to control value (1-4 day) followed by a large rebound (3 fold increase 7 day postdose). These results suggest that increase in the release of pituitary ACTH may be responsible for the increase of adrenal corticosterone release after chlordecone treatment. The increase in the pituitary-adrenal activity is consistent with morphological observations which indicate that adrenal cortical cells and corticotrophs of the pituitary hypertrophy after chlordecone exposure. In an attempt to determine whether chlordecone-elicited increase in the pituitary-adrenal activity could be mediated through the CNS, we measured the turnover of various neurotransmitters. Chlordecone increased the turnover of norepinephrine (NE) and serotonin in the hypothalamus. To further test the possibility that the hyperactivity of the NE and 5-HT systems may be involved in chlordecone-elicited increase in the release of ACTH and corticosterone, phenoxybenzamine and pizotifen (a 5-HT receptor blocker) were employed. Pretreatment with these two receptor blockers completely prevented chlordecone-elicited increase in plasma levels of ACTH and corticosterone suggesting that the hypothalamus may mediate the neuroendocrine effects of chlordecone on the pituitary-adrenal axis.

For future work, we plan to study the molecular mechanism underlying the membrane effects of organochlorine insecticides. Wu et al., (1980) reported that DDT caused repetitive firing in lobster axon presumably by holding the sodium channel open. It is possible that the DDT-induced increase in neuronal firing may be related to increases in turnover of 5-HT or NE (Hrdina et al., 1975). We plan to study the biochemical effects of chlordane, DDT, and mirex on the sodium channel preparation from rat brain. In collaboration with Dr. Robert Aronstam at the University of Georgia, we will investigate the effect of these three compounds on the sodium channel by evaluating their potency to compete for the binding of ^3H -batrachotoxin (a specific ligand for sodium channel protein) to sodium channel.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Elucidation of the neurochemical mechanism underlying organochlorine insecticides-elicited tremor may help us to learn more about the central regulation of tremor. This type of information may also provide the basis for clinical treatment of patients exposed to these insecticides.

PUBLICATIONS

Hong, J.S., Tilson, H.A., Uphouse, L.L., Gerhart, J., Wilson, W.E., and Hunter, V.: Effects of chlordane exposure on the brain neurotransmitters: Possible involvement of striatal serotonin system in chlordane-e-elicited tremor. Toxicol. Appl. Pharmacol. 73: 336-344, 1984.

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Rosecrans, J.A., Hong, J.S., and Tilson, H.A.: The effects of chlordane (Kepone) on pituitary-adrenal-function in the adult Fisher-344 rat. Neurobehav. Toxicol. (In Press).

Tilson, H.A., Hong, J.S., Gerhart, J.M., and Walsh, T.J.: Animal models in neurotoxicology: The neurobehavioral effects of chlordane (Kepone). In: Advances In Behavioral Pharmacology. (In Press).

Herr, D.W., Hong, J.S., and Tilson, H.A.: DDT-induced tremor in rats: Effects of pharmacological agents. Psychopharmacology, (In Press).

Hudson, P.M., Chen, P.H., Tilson, H.A., and Hong, J.S.: Effects of p,p'-DDT on the rat brain concentrations of biogenic amine and amino acid neurotransmitters and their association with p,p'-DDT-induced tremor and hyperthermia. J. of Neurochem. (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 90041-01 LBNT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sensitivity to Amino Acids in Mouse Spinal Cord Neuron Culture

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Clifford L. Mitchell	Pharmacologist	LBNT	NIEHS
Others:	G. Westbrook	Senior Staff Fellow	LDN	NICHD
	P. G. Nelson	Chief	LDN	NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Membrane Physiology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:

1

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

L-glutamate (GLU) and L-aspartate (ASP) are neurotransmitter candidates at primary afferent and intraspinal synapses in the mammalian spinal cord. Several receptor types exist for the excitatory amino acids based on selective activation by kainate (KA), quisqualate (QA) and N-methyl-D-aspartate (NMDA), whereas GLU can activate both NMDA and non-NMDA (i.e. either QA or KA) receptors. Recent evidence in culture suggests that a non-NMDA receptor mediates monosynaptic EPSPs formed between dorsal root ganglion and dorsal horn neurons as well as between spinal cord neurons. The purpose of the present study is to test the chemosensitivity of spinal cord neurons to the above mention amino acids during the first week in culture. The result indicate that at day 2-3, most spinal cord neurons are sensitive to QA (10 μ M) and GLU (100 μ M) as well as γ -aminobutyric acid (GABA, 100 μ M). Responses to KA (10 μ M) are small or absent. QA and GABA responses are associated with conductance increases while GLU responses result in little or no apparent conductance change. Under voltage clamp GLU-activated currents have a region of zero or negative slope conductance consistent with a mixed agonist action on both NMDA and non-NMDA receptors. Both QA and GLU-activated currents have reversal potentials near 0 mV. GABA responses reverse at -50 mV with KMeSO₄ and at 0 mV with CsCl solutions in the patch electrode, consistent with a chloride conductance. By day 7, spinal cord neurons are highly sensitive to all agonists tested. These results suggest that both NMDA and non-NMDA receptors are present before detectable synaptic activity, but that sensitivity increases during a period of rapid synapse formation.

PROJECT DESCRIPTION

OBJECTIVE: Evidence that several receptor types exist in the nervous system for excitatory amino acids is based on selective activation by kainate (KA), quisqualate (QA) and N-methyl-D-aspartate (NMDA). L-Glutamate (GLU) and L-aspartate (ASP) are putative neurotransmitters at primary afferent and intraspinal synapses in the mammalian spinal cord. ASP activates NMDA receptors whereas GLU appears to activate both NMDA and non-NMDA (i.e. either QA or KA) receptors. However, little is known concerning the onset of sensitivity to these amino-acids in developing spinal cord neurons in tissue culture.

Our objective is to characterize the development of these receptors and to determine the relationship of this development to synaptic activity.

METHODS EMPLOYED: Neurons were dissociated from 13 day embryonic mice and plated on collagen-coated 35 mm dishes at 6×10^5 cells per plate. Growth medium contained 5% horse serum and a nutrient supplement. Whole-cell patch₂ recordings were made in physiological saline containing 2 mM Ca^{2+} and 1 mM Mg^{2+} . Agonists were dissolved in recording solution and pressure ejected from pipettes near the soma. Under these culture conditions, neurite outgrowth begins within 24 hours and Na^{+} -dependent action potentials can be evoked with intracellular stimulation. However no detectable spontaneous EPSPs or IPSPs ($< 200 \mu\text{V}$) are present for the first 2-3 days, then spontaneous synaptic activity develops rapidly between days 4 and 7 (Westbrook, et. al., Soc. Neurosci Abs. 9, 505, 1983).

MAJOR FINDINGS AND PROPOSED COURSE: At day 2-3, most spinal cord neurons were sensitive to QA (10 μM) and GLU (100 μM) as well as γ -aminobutyric acid (GABA, 100 μM). Responses to KA (10 μM) were small or absent. QA and GABA responses were associated with conductance increases while GLU responses resulted in little or no apparent conductance change. Under voltage clamp GLU-activated currents had a region of zero or negative slope conductance consistent with a mixed agonist action on both NMDA and non-NMDA receptors. Both QA and GLU-activated currents had reversal potentials near 0 mV. GABA responses reversed at -50 mV with KMeSO_4 and at 0 mV with CsCl solutions in the patch electrode, consistent with a chloride conductance. By day 7, spinal cord neurons were highly sensitive to all agonists tested.

These results suggest that both NMDA and non-NMDA receptors are present before detectable synaptic activity, but that sensitivity increases during a period of rapid synapse formation.

Future research will examine, in detail, the contribution of synaptic activity on the increase in sensitivity to these amino-acids. This will be done by (1) blocking synaptic activity using tetrodotoxin, (2) use of specific antagonists for the various amino-acids and (3) enhancing synaptic activity by picrotoxin and/or bicuculline.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The properties of the nervous system that are considered central to its function are the

excitability of its elements and the communication of excitability changes among its elements. The results of this research should contribute to our understanding of the developmental aspects of these properties and provide insight into factors essential for establishing synaptic activity and its modulation.

TITLE: USE OF HOMECAGE BEHAVIORS TO SCREEN FOR POTENTIAL TOXICITY OF CHEMICALS

CONTRACTOR'S PROJECT DIRECTOR: HUGH EVANS, PH.D.

PROJECT OFFICER (NIEHS): HUGH A. TILSON, PH.D., HEAD
NEUROBEHAVIORAL WORKGROUP, LBNT

DATE CONTRACT INITIATED: MAY 25, 1982

CURRENT ANNUAL LEVEL: \$113,294

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this research are: 1) to obtain information about the utility of continuous home-cage monitoring of domiciliary behavior as a reliable index of neurobehavioral toxicity, 2) to determine the effects of representative neurotoxicants on these behaviors, and 3) to attempt to correlate alterations in tissue levels with the presence and magnitude of behavioral effects.

METHODS EMPLOYED: A homecage monitoring system is used to measure continuously food and water ingestion and spontaneous motor activity. Rats are dosed chronically with toxic agents and alterations in the frequency and cyclicity of these behaviors are determined using computer-assisted programs. At the end of the dosing periods, animals are sacrificed to obtain blood and brain levels of the compound.

MAJOR FINDINGS AND PROPOSED COURSE: A system to monitor up to 40 rats was validated by demonstrating that growth curves for rats maintained with the system are identical to those maintained with conventional watering and feeding and that the rat's diurnal cycle in activity and ingestive behavior shifts in response to change in the daily light-dark cycle. Expected changes in activity and consummatory behavior occurred following food deprivation and post-deprivation rehabilitation. The systemic administration of various psychoactive drugs (d-amphetamine, scopolamine, methylscopolamine) also demonstrated the sensitivity of the system to detect behavioral changes produced by these agents.

Diurnal patterns of feeding, drinking and locomotor activity were measured for 2 weeks after a single oral dose of trimethyltin (0, 3, 5, or 7 mg/kg). Body weights and feeding and drinking efficiency ratios were also determined daily. TMT caused a dose- and time-related decrease in body weight which was associated with decreases in food consumption. Water consumption increased two-fold immediately after TMT and was elevated up to 2 weeks postdosing. The diurnal patterns of drinking and rearing were disrupted by all doses of TMT, i.e., the normal peak in rearing activity normally occurring immediately prior to light onset was attenuated after all doses of TMT. The data show that the regulation of food and water intake is compromised in a time- and dose-related fashion after TMT and that the rat's cyclical patterns of activity were affected by doses lower than those required to affect food and water intake.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: One of the earlier signs of toxicity is a change in body weight. The system being developed and validated will permit the quantification of changes in ingestive behaviors and motor activity preceding body weight alterations. The use of behavioral indices of toxicity may prove to be an early warning for the detection of toxicity, as well as providing clues to understand how toxicants interfere with neurobiological processes controlling food and water intake.

NORTH CAROLINA STATE UNIVERSITY - Raleigh, NC
(NIH-N01-ES-2-5019)

TITLE: Research Support to Investigate Teratogenic Effects of Environmental Agents Using Avian Species

CONTRACTOR'S PROJECT DIRECTOR: Carmen Parkhurst, Ph.D.
J. Paul Thaxton, Ph.D.

PROJECT OFFICER (NIEHS): Donald I. McRee, Ph.D., Research Physicist
Laboratory of Behavioral and Neurological Toxicology

DATE CONTRACT INITIATED: August 1, 1982

CURRENT ANNUAL LEVEL: \$160,015

PROJECT DESCRIPTION

OBJECTIVES: The objectives of the contract are to provide housing and maintenance services for care of research animals (Japanese quail) and to provide support staff necessary to conduct biological tests on birds which have been exposed to environmental agents.

METHODS EMPLOYED:

The contract will provide a special breeding colony for adequate supply of fertilized Japanese quail eggs, brooding facilities for housing and care of baby quail, and housing and maintenance of experimental Japanese quail throughout duration of experiments. The contractor will also provide support personnel, materials, supplies, facilities and equipment for conduct of biological experiments. The support personnel will include a full-time professional level researcher, two full-time technical support persons, and two part-time caretaking and maintenance support persons. Research protocols will be developed by the staff of the Laboratory of Behavioral and Neurological Toxicology (LBNT) of NIEHS with the participation of the professionals provided by the contractor. The research projects will then be conducted by the personnel provided by the contractor with consultation and assistance of the staff at NIEHS.

MAJOR FINDINGS AND PROPOSED COURSE:

Fifteen research protocols have been written by the staff of NIEHS and N.C. State University, and the research have either been completed or is in progress. Some of the protocols are concerned with the biological effects of microwave radiation and the other protocols are studies to determine the effects of chemical agents. The microwave bioeffect studies are designed to determine the response of quail to various stressful conditions after microwave exposure during embryonic development as the quail mature. In all cases the exposures were at a microwave frequency of 2.45 GHz, at an incident power density of 5 mW/cm² (specific absorption rate=4.03 mW/g) and during the first 12 days of development. The hemetological response of immunized quail was measured in juvenile (5 weeks old) and adult (22 weeks old) quail at 3, 6, and 9 days post-immunization. Only the exposed juvenile female quail elicited a response different from controls. Total reticulocytes were initially depressed and at 9 days post-immunization they expressed an increased number of reticulocytes in comparison to controls. The response of quail

exposed to microwave radiation in the above manner to hemorrhagic stress was investigated. The quail were mechanically hemorrhaged to 70% of their calculated blood volume. Circulating erythrocyte and reticulocyte numbers were depressed below controls by 24 hr post-hemorrhage in exposed juvenile males. However, lymphocyte numbers elevated in exposed juvenile males at 72 hr. These data suggest that hematopoiesis is affected by embryonic irradiation and that the effect persists for a long time after hatching (exposure).

Japanese quail eggs were injected with DES (0.9-1000ug) dissolved in 50 ul of corn oil on day 1 of incubation. Higher doses of DES (250-1000ug) reduced hatchability to 37-33% compared to 61% for corn oil injected controls. Lower doses of DES (0.9-125ug) had no effects on hatchability. In a second study, eggs were injected with 0.9 or 1.9 ug of DES and the survivors were assessed up to 12 wks posthatch. DES did not affect hatchability, but did increase mortality during the first 4 wks posthatch. Females were affected more than males. At 10 days of age, open field activity of some birds was examined. The acquisition and reversal of a visual discrimination task was studied at 6 wks of age. Exposure to DES blocked egg production in these birds. The oviduct weights of 12 wk old females were decreased by 50%, but ovarian weights were not affected. Testicular weights were not affected. In a third study reproductive behaviors and social-dominance behaviors of males were markedly attenuated in birds exposed to 0.48 or 1.9ug DES in ovo. Studies to determine the effect of chlordecone (Kepone) on embryonic mortality, survival, morphology and reproduction in Japanese quail were begun. Results show that 1.0 mg/egg of chlordecone injected on day 1 of incubation induced tremors at hatching and caused a 66% mortality at 5 weeks of age.

The projects planned for next year includes the effects of embryonic exposure to microwaves on reproduction. Egg production, fertility, hatchability and monthly mortality will be measured. In addition, the effects of microwave exposure during embryonic development or quail behavior at adulthood will be investigated. Experiments to determine threshold level effects of chlordecone (Kepone) on mortality, survival, and reproduction will continue. In addition, studies to determine the effects of embryonic exposure to DDT will be started in the next year.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The results of this research will increase our knowledge of the biological effects of microwave radiation and chemical agents. The use of the avian embryo as a test system provides the capability of studying the direct effects of environmental agents on development without maternal complications.

PUBLICATIONS

Gildersleeve, R.P., Tilson, H.A., and Mitchell, C.L.: Injection of diethylstilbestrol (DES) on the first day of incubation affects morphology of sex glands and reproductive behavior of Japanese quail. Teratology 31: 101-109, 1985.

Gildersleeve, R.P., Thaxton, J.P., Galvin, M.J., and McRee, D.I.: Hematological response of immunized and serially bled Japanese quail. Poultry Science, 1985, in press.

Gildersleeve, R.P., Phelps, P.V., Thaxton, J.P., and McRee, D.I.: Effects of phlebotomy on reticulocyte numbers in Japanese quail. Poultry Science, 1985, in press.

LABORATORY OF GENETICS

LABORATORY OF GENETICS

Summary Statement

Mutagenesis: A primary objective of the NIEHS is to develop the science base required for protecting humans against the genetic ill effects of environmental agents. The corresponding objective of the Mutagenesis Section is to explore mechanisms of mutagenesis. This is pursued by the development and application of model systems ranging from DNA polymerases in the test tube through mammalian cells in culture.

The months surrounding FY-85 have seen a considerable number of staff changes in the Section. Drs. Glickman and Ripley departed for senior university positions, having conducted major programs here for several years. Dr. Volkert will depart at the end of the year. Two scientists formerly in the Glickman group Drs. de Jong and Schaaper, have gained a considerable level of independence for the remainder of their NIEHS tenures and are now functioning as lead investigators. Dr. Sugino, who had left the Section earlier for a position at a university, rejoined us at the beginning of the year to head a major group. Dr. Kenneth Tindall will join us at the end of the year to initiate a program in mammalian somatic cell mutagenesis.

The Drake group concentrates on the analysis of mutagenic mechanisms using bacteriophage T4 as a model system. The main recent focus has been upon error-prone repair, the main mutagenic response to chemicals and radiations in most organisms. The functions of at least three genes, and probably several more, are required for this response; defects in these genes not only abolish mutagenesis but also reduce survival and may affect several other aspects of viral reproduction. Several years ago, this group isolated mutations of two of these genes, which differentially affected endpoints such as mutagenesis and survival. These mutant genes are now being cloned into expression vectors so that their proteins can be examined biochemically to determine how they have been altered. At the same time, similar mutations are being isolated from a third gene of this system. During the course of these studies, a mutation in the gene that encodes the major viral DNA-binding protein was discovered to engender increased sensitivity to inactivation by ultraviolet irradiation or methyl methanesulfonate treatment. A major portion of this increased sensitivity was found to arise in a manner independent of the two major viral DNA repair systems (excision repair and post-replication recombinational repair, the latter also encompassing error-prone repair). Thus, T4 encodes three, not two, major mechanisms for survival after DNA damage.

The Schaaper group, nominally a part of the Drake group, actually functions quite autonomously. They have, in this first year, developed two systems for the mixed in vitro/in vivo analysis of mutation. The systems depend upon highly defined mutational targets embedded in phage vectors and upon the processing of DNA damage in these targets either by intact cells or by cell extracts. An extensive spontaneous mutational spectrum has been determined for one of these systems, against which induced spectra can now be compared. Using the other system, they have explored the effects of dNTP pool biases upon replicational fidelity.

The Kunkel group concentrates on the role of defined enzymatic systems in determining the fidelity of DNA synthesis *in vitro*. They have developed and extensively applied a phage-borne mutational target that can be replicated *in vitro*, with or without DNA damage, and then expressed *in vivo*. (While developing this system, a new method of site-directed mutagenesis was developed and patented. This method has been widely adopted both commercially and in institutional laboratories, and is the best available in many circumstances.) To date, this system has been used to characterize the qualitative and quantitative aspects of polymerase fidelity using the three main vertebrate polymerases (the nuclear replicase, the nuclear repair polymerase and the mitochondrial replicase). These enzymes, all lacking associated exonucleolytic proofreading functions, nevertheless replicate DNA with fidelities that often exceed those expected from thermodynamically passive systems, thus showing that the enzymes take active roles in discriminating among incoming dNTPs. A remarkable correlation has also been discovered between accuracy and processivity (the average number of nucleotides incorporated by a polymerase per association with a DNA template/primer). Furthermore, since the system includes precise DNA sequence determinations of all of the mutants, extensive information has accumulated about the kinds of errors made by these polymerases. Certain error classes had not been previously recognized. For instance, one error class is best modelled by allowing the polymerase to detach from its template but remain bound to its primer, then to move transitorily to a new template sequence. Another error class is well modelled by allowing the polymerase to read one base ahead of the intended site, generating either frameshift or base substitution mutations depending upon whether the correct replication frame is resumed. This system has also been applied to the analysis of the fate of DNA damages. Abasic sites, for instance, have been shown to be highly mutagenic, providing that the host cell's capacity to conduct error-prone repair has been induced; and deaminated cytosine has been found to be highly mutagenic, providing that the host cell's capacity to excise such damage has been reduced.

The Sugino group studies eukaryotic DNA replication and repair, mainly but not exclusively in yeast and mainly but not exclusively using *in vitro* systems. A powerful model system involving yeast plasmids as replication templates has been used to chase down enzymes important to DNA synthesis. To date, for instance, the system has been used to identify, purify and characterize the yeast DNA replicase and a number of proteins that enhance its activity, including a separate DNA primase, three DNA-binding proteins, two RNase H activities and a DNA-dependent ATPase that exhibits DNA-unwinding activity and appears to be the product, or under the control of, the RAD3 gene. In major parallel efforts, mutants potentially defective in DNA replication have been discovered in 30 complementation groups and have been partly mapped, while the cloning of the genes encoding the various polymerase and accessory proteins is proceeding steadily. Thus, the entire molecular genetics of DNA replication in yeast is coming under study and, in a joint project with the Kunkel group, will soon be explored for its fidelity determinants. In another yeast project, the proteins required for recombination are being sought by their ability to catalyze DNA rearrangements *in vitro*; preliminary work has focused on the product of the recombination gene RAD52. Finally, as an adjunct to the yeast system that may eventually extend much of its power to the analysis of mammalian DNA replication, an SV40 *in vitro* DNA replication system is being established. DNA topoisomerases I and II, DNA primase, DNA-dependent ATPase, and RNase H have been purified from human and calf cells, and their efficacies in the SV40 system

are being explored. During this work, it was discovered that the type I topoisomerase expresses a novel "reverse gyrase" activity. The SV40 system, too, is being adapted for studies on fidelity.

The de Jong group, nominally a part of the Sugino group, actually functions somewhat autonomously. Continuing a project initiated before the departure of Dr. Glickman, they have worked to establish and apply a system for determining mutational specificities in mammalian cells. Starting from an *aprt*-hemizygous cell line, numerous fully *aprt*-deficient mutants were obtained (100 of spontaneous and 60 of X-ray-induced origin). Vectors have been developed to recover these mutant sequences in a form suitable for DNA sequence analysis and many of the mutants have been isolated and characterized.

The Volkert group is pursuing two aspects of DNA repair and mutagenesis in *E. coli*. The *recF* gene is important in certain aspects of recombination and mutagenesis, but its role has remained rather mysterious. Recently, evidence has accumulated to support the notion that it plays a crucial role in converting the product of the *recA* gene from its inactive to its proteolytically active state following, for instance, UV irradiation. The current major effort of this group, however, is now focused on mechanisms by which cells protect themselves from chronic exposures to alkylating agents, particularly by the induction of repair systems. In addition to the classically described system, inducible genes have been discovered that reveal a level of complexity not previously guessed, including considerable specificity for the kind of alkylating agent (even among simple methylators). For some of these genes, transcription is promoted by alkylation treatments but their activity seems not be required for full protection against the lethal effects of alkylation.

Eukaryotic Gene Structure: Members of this section are studying the genetic architecture of higher organisms from a variety of viewpoints. Selected genes are being studied genetically and molecularly to understand their organization, function, regulation and evolution. Particularly important is the advancement of the understanding of how genes are regulated during development. Considerable effort is also directed toward determining the nature and amount of genetic variability that exists in populations of organisms and the factors that influence this variability in both qualitative and quantitative terms.

The Langley and Aquadro groups are involved with experimental and theoretical studies of genetic variation in natural populations. One approach they have taken to these questions is to survey lines of *Drosophila melanogaster* isolated from natural populations. Selected genes and regions surrounding them have been scored for restriction site polymorphisms. For example, an 80 kilobase region around the gene (*Ddc*) encoding dopa decarboxylase was surveyed among forty-six *Drosophila* lines isolated from natural populations. Of 84 restriction sites scored, 24 were polymorphic. The polymorphic sites are scattered throughout the 80 kb region but those that are highly variable are clustered in a 40 kb region containing *Ddc* and a dense cluster of lethal-mutable genes. In similar fashion 10 inserts, presumably of transposable DNA sequences, were localized in the same 40 kb region while in the adjoining 40 kb where only one gene is found no inserts were seen. The marked non-random distribution of variable sites suggests that the patterns may reflect selection for favored combinations of alleles in *Ddc* and the group of eight lethal-mutable genes. Surprisingly no consistent pattern of association between restriction site haplotype and *Ddc* activity is apparent. In fact two lines having inserts, one of 5 kb within an

intron of Ddc and the other 1.5 kb at the 5' end of Ddc both show completely normal enzyme activities. This is quite unlike the picture that emerged from similar surveys of these same lines for the region around the gene encoding alcohol dehydrogenase (Adh), where there is a strong indication that sequence variants have modified Adh activities. Nearly 200 kb additional have been assayed around the white, rudimentary, Notch, amylase and amylase pseudogene loci. Here again there are no obvious correlations between haplotypes and the activities of these wild-type genes. Transposable elements were found at any one site only rarely, a result consistent with their presence being slightly deleterious.

Dr. Aquadro has accepted a university appointment where he will continue his studies in molecular population genetics.

Dr. Kreitman working with the Langley group has developed a technique that allows identification of essentially all haplotypic variation at selected gene loci. It involves a multi-level analysis of variation. First is the identification of restriction site polymorphisms using endonucleases that have four-nucleotide sequence specificities. The specific haplotypes are then examined by direct cloning and nucleotide sequencing. This detailed analysis is being applied to two loci, Adh and white in lines of D. melanogaster and to Adh in D. simulans.

It is clear that a large fraction of spontaneous mutations in Drosophila is associated with transposon insertion/deletion. To determine the generality of this observation, the Langley group has isolated forty independent mutations showing deficiency of hypoxanthine-guanine phosphoribosyl transferase activity in human fibroblast cells. These mutants are now being examined with molecular probes to assess the involvement of transposable elements and other types of DNA rearrangements in spontaneous mutations in humans.

Two mutator systems involving transposable elements are being studied in the laboratory. The Langley group has isolated clones of the transposon tom from a strain of D. ananassae that exhibit unusual properties. The mutator was detected by C. Hinton through its ability to cause mutations at loci involved with eye morphology. Hinton described 21 loci where ocular morphology (Om) mutations occurred, all of which have similar phenotypes. Dr. Langley cloned a 6.5 kb insert from the mutable strain that is found to be completely linked to the Om loci. The insert is a transposable copia-like element. This system is being further studied to determine why tom seems to have such high specificity for loci affecting eye structure.

The other mutator system was described by J. Lim in a strain of D. melanogaster designated Uc (unstable chromosome). The strain exhibits very high rates of mutation, chromosome rearrangements and sterility. The major causative agent here has been determined to be a transposon called gypsy, a retrovirus-like element similar to copia. The Uc strain has up to 65 copies of this element per diploid chromosome set, while stable strains usually have fewer than 10 copies. When the gypsy-laden strain is crossed to a gypsy-free strain, the F₁ hybrid females exhibit high rates of mutation, as do their offspring. F₁ males on the other hand are mutationally stable. It has been determined that gypsy is mobilized during the early embryonic cell divisions, increasing in copy number and inserting into new chromosome sites. Mutations that occur in germ line cells usually then appear as clusters of mutant offspring. The gypsy mutator system

is quite different from the other dysgenic systems (P-M and I-R) found in *Drosophila*. The origin of the high copy number of gypsy in the Uc strain and the factors involved in the increase are being investigated. Since endogenous proviral sequences of retroviruses are so prevalent in vertebrates including humans, it is important to understand the conditions under which increased copy number and mobility of retrotransposons are stimulated.

One of the genes that mutates at a very high rate by insertion/deletion of gypsy is the cut locus, being studied by J. Jack. He has cloned DNA sequences encompassing the gene, which is unusually large, measuring about 200 kb. The cut locus is of great interest because it is regulated during development in both temporal and tissue specific patterns. Dr. Jack has analyzed a large number of mutants and determined that those affecting development primarily in legs result from deletions of the most centromere-distal segments of the gene. Mutations in the central portions of the gene cause primarily wing defects while in the centromere-proximal section are found sequences that mutate to lethal states and affect wings, head and thorax. The organizational complexity of the very large cut gene is matched by its developmentally complex program. Dr. Jack is now studying the transcription of RNA from this gene to learn about specificity of regulation in this complex unit.

The Boswell program also deals with an important aspect of developmental genetics, the mechanism for germ cell determination and differentiation. Dr. Boswell's approach to these problems is through the analysis of mutations of *Drosophila* that affect primordial germ cell formation. Because the determination of the fate of embryonic cells is regulated by morphogenetic determinants localized in the ooplasm, genes that are involved in these developmental programs often exhibit maternal effects. A female of mutant genotype fails to synthesize a necessary cytoplasmic factor for germ cell differentiation, resulting in offspring that are sterile. Dr. Boswell has identified a number of such grandchildless mutants and has concentrated on one locus called tudor. The germ plasm produced by six different alleles of tudor has been analyzed by electron microscopy. These mutants affect the amount of polar granule material, having either granules reduced in size or no granules. All fail to form germ cells. The genetic and cytological analyses of the locus have progressed to the point where molecular cloning is underway. The cytological position of tudor in the polytene chromosomes has been determined and from cloned segments near by, Dr. Boswell has walked about 60 kb toward the locus. The understanding of the mechanisms of determination and differentiation of germ cells should be advanced considerably through the study of this and related maternal effect genes.

The Judd group has been studying alleles of the white locus that are changed in their ability to regulate normally during development. The w^{zm} allele has wild-type function except when coupled with the mutation z⁻, in which case it exhibits a mottled mosaic phenotype of normal and mutant cells in the eyes of the fly. This mutation is caused by the insertion of the transposon BEL into the first intron near the 5' end of the gene. The transposon has been cloned and mapped by restriction endonucleases. w^{zm} is mildly unstable and has produced a family of derivatives some of which like w^{zm} have mosaic phenotypes that are clonal and autonomous in their cellular expression. Another group of w^{zm} derivatives, associated with the transposition of the entire white region and some adjoining genes into the third chromosome, show mosaicism that is nonclonal and nonautonomous. Some of these are wild-type in the z⁺ background,

assuming a mutant phenotype only in the presence of z_1^1 . The interaction of the w and z loci is further complicated by the fact that expression of white is dependent on chromosome structure. Chromosomal rearrangements that upset the somatic pairing of the homologous chromosomes containing the white locus return the phenotype of z_1^1 individuals to wild-type. This trans-regulatory phenomenon is believed to be a reflection of what normally is a cis-acting system responsible for the control of white locus function. By determining the basis for the transvection, it is hoped that clues will be found leading to understanding the normal regulatory mechanisms.

The works of two other groups, Drs. Voelker and Searles focus on still other aspects of gene structure and regulation. Dr. Searles has cloned the gene that encodes tryptophan oxygenase (vermilion) and has characterized its structure and that of a number of different alleles. Some of the vermilion alleles are due to the insertion of transposons into the gene. Of considerable interest is the observation that some of these alleles are suppressible by mutations at the suppressor of sable locus (su-s). Dr. Voelker's group has been studying the su-s gene; they have cloned it and characterized a number of mutants. A ~5 kb RNA homologous to about 8 kb of the su-s region has been identified and the gene has been mapped by S1 protection mapping. This DNA sequence is now being placed in an expression vector in order to obtain and characterize the protein gene product. One of the objectives of these programs is to determine how suppression of transposon-induced mutations is carried out by the mutant forms of the su-s gene.

The structure, expression and mutation of mammalian genes are being studied by four groups in the section. Dr. S. S.-L. Li has been investigating the multigene family encoding lactate dehydrogenase proteins (LDH). LDH-A genomic clones containing a functional gene and several pseudogenes from mouse and human have been isolated and sequenced. Li has shown that the protein-coding sequence of LDH-A genes of both mouse and humans is interrupted by six introns. The intron/exon boundaries appear to be near the ends of secondary structural domains of the folded polypeptide chain or near proline or hydrophobic amino acids at random-coil regions. In the monomeric subunits, the intron/exon junctions are located on the surface, although the third and fourth intron junctions are at internal positions in the tetrameric LDH-molecules. From a mouse genomic clone of LDH-A, nucleotide sequencing shows the positions of initiation sites of transcription and translation, and the regulatory sequences including TATA box, CAAT box, hexanucleotides CCGCCC and the cAMP-responsive sequence. In addition, Dr. Li is examining the tumor-associated LDH-K isozyme and has shown it to be a phosphotyrosine-containing LDH-A4 protein.

The Skow group continued its study of genes encoding α -, β - and γ -crystallins of the mouse eye lens. Analysis of α A-crystallin sequence variants have located the α A gene in chromosome 17, very close to the major histocompatibility region H-2K. Linkage disequilibrium is found for Acry-1 alleles and H-2 haplotypes, suggesting that Acry-1 should be considered to be a component of the MHC. No polymorphisms have been detected for the β -crystallin sequences but analysis of DNA from mouse x hamster somatic cell hybrids show β cry-1 located on chromosome II. Considerable variation is found in the γ -crystallin sequences, which allows mapping of the locus to chromosome 1 very near the anophthalmic-producing, Elo locus.

Dr. Skow has accepted an academic appointment where he will continue his studies of heritable lens disfunction.

Dr. Johnson's group is studying spontaneous and induced mutations in mice. A large number of ethylnitrosourea induced mutations has been recovered. They were recognized as electrophoretic variants among proteins encoded by about 30 different loci. Spontaneous mutations are also picked up by the screening process. One of the disorders discovered is a β -thalassemia resulting from a deficiency. Animals derived from the electrophoretic studies were also examined for morphological variation in the skeleton. Quantitative and qualitative measures of variation are being taken among mutagen treated and untreated groups. Correlations will be made between mutation rate, as measured by recovery of electrophoretic variants, the amount or type of skeletal variants.

Dr. Malling's group is involved in the development of systems that will detect mutations in single mammalian cells. Several approaches seem promising. Malic enzyme has been purified and antibodies raised against it. The antibodies have been used to demonstrate that they will react to sperm and somatic cells. A mutant form of MOD-1 has also been purified and tests will be done to determine whether specific changes in enzyme structure can be detected in sperm or in heterozygous cells using the antibody reaction assay. Similar methods are being used to develop LDH-A as a marker locus. Φ X174 DNA has been used to transform mouse L-cells and techniques for recovering these sequences and transfecting into spheroplasts are being worked out. The idea is to assay multiple copies of the viral DNA for changes induced by mutagen treatment in a way that allows all of the mammalian physiological factors to operate in the mutagen response. Scoring for the mutagen-induced changes would then be done directly on each viral sequence. Similarly a system for monitoring mitochondrial DNA damage is being explored.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 60099-06 LG																
PERIOD COVERED October 1, 1984 to September 30, 1985																		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Organization-regulation of mammalian lactate dehydrogenase genes																		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 40%;">Steven S.-L. Li</td> <td style="width: 30%;">Research Geneticist</td> <td style="width: 15%;">LG, NIEHS</td> </tr> <tr> <td>Others:</td> <td>Kayoko Fukasawa</td> <td>Visiting Fellow</td> <td>LG, NIEHS</td> </tr> <tr> <td></td> <td>Fu-zon Cung</td> <td>Visiting Fellow</td> <td>LG, NIEHS</td> </tr> <tr> <td></td> <td>Uma Bhattacharyya</td> <td>Visiting Fellow</td> <td>LG, NIEHS</td> </tr> </table>			PI:	Steven S.-L. Li	Research Geneticist	LG, NIEHS	Others:	Kayoko Fukasawa	Visiting Fellow	LG, NIEHS		Fu-zon Cung	Visiting Fellow	LG, NIEHS		Uma Bhattacharyya	Visiting Fellow	LG, NIEHS
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	Fu-zon Cung	Visiting Fellow	LG, NIEHS															
	Uma Bhattacharyya	Visiting Fellow	LG, NIEHS															
COOPERATING UNITS (if any)																		
LAB/BRANCH Laboratory of Genetics																		
SECTION Eukaryotic Gene Structure Section																		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																		
TOTAL MAN-YEARS: 3.3	PROFESSIONAL: 3.0	OTHER: 0.3																
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input checked="" type="checkbox"/> (b) Human tissues</td> <td><input type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews									
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<input type="checkbox"/> (a2) Interviews																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>LDH-A cDNA clones from mouse and human have been isolated and sequenced. Mouse pMLA 73 was found to contain the 393bp of coding region and 496 bp 3' untranslated sequence including poly (A) tail. Four human cDNA clones consist of sequences of approximately 1,700bp, that is, the complete coding sequence (999bp), the 5' (97bp) and 3' (565bp) untranslated regions. Several LDH genomic clones have also been isolated and partially characterized from mouse and human DNA libraries. Mouse genomic clone M15 appears to contain a functional gene of at least 8Kb consisting of seven exons and six introns. The relationship between the exon-intron organization of LDH-A gene and the structural-functional domains of LDH-A protein are illustrated. The nucleotide sequence of a human LDH-A pseudogene has been determined. 12.9% nucleotide differences were found between the pseudogene and LDH-A cDNA, and their significant implications on mammalian mutations and molecular evolution are observed. It is of interest to elucidate the genetic mechanism(s) underlying the tissue-specific expression of three LDH genes in human and other vertebrates.</p>																		

PROJECT DESCRIPTION

PROBLEM: Structural organization, regulation of expression and molecular evolution of a mammalian multigene family coding for lactate dehydrogenase proteins are being studied to understand the role of the several polypeptide domains in the function and evolution of these proteins.

OBJECTIVES: In mammals lactate dehydrogenase isozymes A₄ (muscle), B₄ (heart) and C₄ (testes) are encoded by three different gene loci and the expression of these three LDH genes is developmentally regulated and tissue-specific. Recently, the LDH isozymes were shown to be ssDNA-binding proteins. The detailed knowledge about the genomic organization of this multigene family will shed light on molecular aspects of their regulation of expression and molecular evolution.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: The genomic clones of three LDH genes from human and mouse have been/will be isolated and their organization and regulation of expression will be illustrated. Analysis of the DNA sequences encoding the proteins and those flanking the structural genes will allow accurate evaluations of genetic mutation events which perturb the regulatory mechanisms as well as those which modify the protein coding sequence itself. In humans the deficiencies of LDH-A or LDH-B subunits have been correlated with different clinical features and the molecular nature of their genetic mutations will be determined.

The dinucleotide CpG deficit appears to be a distinctive feature of mammalian DNA and the methylated CpG sequences appear to control the tissue-specific gene expression. The extent of DNA methylation at the regulatory region of the human LDH genes will be examined by the genomic blotting of placenta and sperm DNAs. The methylated CpG sequences have also been suggested to be mutational hotspots in mammalian DNA and the high frequency of polymorphism at Msp I and Tag I restriction sites containing the CpG sequence have been observed in human DNA.

The expression of the LDH genes is regulated by dibutyryl cAMP and isoproterenol as well as the stress factors such as heat-shock, anaerobic-shock (anoxia, hypoxia), oxidative inhibitors, oxidizing chemicals, etc. The cellular level of nucleotide pool such as ATP/ADP, NAD⁺/NADH, AppppA, etc. may play an important role as signaling 'alarmones'. Recently, LDH-K isozyme is found in many carcinomas and in cultured cells transformed by the Kirsten murine sarcoma virus. The expression of this LDH-K seems to be induced by anaerobic shock. The molecular basis for the expression of the LDH-K and its possible relationship to the cancer will be studied.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: A mouse LDH-A cDNA clone pMLA73 was isolated and it was found to contain the 393 bp of protein-coding sequence and 496 bp of the 3' untranslated region including a poly (A) tail. The pMLA cDNA was used as a probe to isolate nine mouse genomic clones and one of them, designated as M15, was shown to contain a functional LDH-A gene. The complete sequence of 12,856 nucleotides present in M15 clone has been determined, demonstrating that the protein-coding sequence is interrupted by six introns.

The relationships between the exon-intron organization of LDH-A gene and the structural-functional domains of LDH-A protein are illustrated. The location of all six introns appears to be either near proline or hydrophobic amino acids at random-coil regions or near the ends of secondary structures. The positions of these introns are located on the surface of monomeric subunits, although those for the third and fourth introns are at internal positions in the tetrameric LDH molecules.

Eight LDH-A cDNA clones have been isolated and characterized from a human fibroblast cDNA library. Four cDNA clones appear to contain nearly full-length inserts and the complete nucleotide sequence of 1710bp consists of the protein-coding sequence (999bp), the 5' (97bp) and 3' (565bp) untranslated regions and poly(A) tail (49bp). Seventeen LDH-A-like clones have also been isolated and characterized from human genomic library. The nucleotide sequence of a processed, intronless LDH-A pseudogene was determined. A comparison between human LDH-A cDNA and the processed pseudogene sequences reveals 114 transitions, 65 transversions and 36 deletions/insertions. Only four out of the 25 CpG dinucleotides present in the cDNA sequence remain unchanged, although the sequences possess 87.1% homology. Further, one of the human genomic clones has been shown to be a functional LDH-A gene.

Using an intron probe from M15 clone, only a single LDH-A functional gene was demonstrated in the total DNA blots from mouse liver. Further restriction mapping and partial nucleotide sequencing of mouse and human genomic clones as well as Southern blot analysis of mouse liver DNA and human placenta DNA appear to suggest the existence of many LDH-like pseudogenes, and this may have significant implications in the mechanisms of their origin and molecular evolution.

FUTURE RESEARCH PLANS: 1. The genomic clones of LDH-B and C functional genes from human and mouse will be isolated and their structure determined by analyses of nucleotide sequence, R-loop and heteroduplex. The potential control (i.e. enhancer) and protein-coding sequences of different LDH genes will be compared and analyzed in order to obtain the information on genetic regulation and molecular evolution.

2. The molecular basis of human LDH-A and LDH-B deficiencies will be investigated by genomic blot analysis using LDH gene probes and, possibly, by sequence analysis of cloned mutant genes. The molecular nature of spontaneous and induced missense mutations at mouse LDH-A gene locus will also be determined.

3. The developmental and tissue-specific expression of mouse LDH-A, B and C genes will be studied by quantitative analysis of their mRNAs using gene-specific probes. The in vitro cell-free transcription system and the in vivo gene expression in cultured cells for human and mouse LDH genes will be developed. The in vitro mutagenesis of LDH genes will be used to study the control of gene expression. In particular, the cAMP-receptor protein recognition sequence of LDH-A gene will be 'mutated' and its effects on the responses to cAMP or isoproterenol will be examined. The LDH-A cDNA clone will also be used to study the structural-functional relationship of LDH protein by site-directed mutagenesis.

4. The extent of CpG methylation at the LDH-A gene will be compared among the DNA samples isolated from different tissues of human and mouse. The nuclease sensitivity of chromatin, especially, the 5' upstream regulatory region of the LDH-A gene, will be studied. In view of its ssDNA-binding ability, the possibility of autoregulation at the transcription and translation levels of LDH genes will be investigated.

5. The precise locations of LDH gene loci, especially LDH-C gene, in human and mouse chromosomes will be determined by in situ hybridization and/or genomic blot analysis of hybrid somatic cells. The possible relationship between the expression of LDH genes and oncogenes (H-ras, K-ras & N-ras) will be investigated.

PUBLICATIONS

Tsujibo, H., Tiano, H.F. and Li, S.S.-L.: Nucleotide sequences of the cDNA and an intronless pseudogene for human lactate dehydrogenase-A isozyme. Eur. J. Biochemistry 147: 9-15, 1985.

Akai, K., Yagi, K., Tiano, H.T., Pan, Y.-C.E., Shimizu, M., Fong, K., Jungmann, R.A. and Li, S.S.-L.: Isolation and characterization of a cDNA and a pseudogene for mouse lactate dehydrogenase-A isozyme. Int. J. Biochemistry (in press), 1985.

Li, S.S.-L., Tiano, H.F., Fukasawa, K., Yagi, K., Shimizu, M., Sharief, F.S., Nakashima, Y., and Pan, Y.-C.E.: Protein structure and gene organization of mouse lactate dehydrogenase-A isozyme. Eur. J. Biochemistry (in press), 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 60145-03 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Mutational Specificity of Purified DNA Replication and Repair Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: T. A. Kunkel Senior Staff Fellow LG NIEHS

Others: A. Sugino Visiting Scientist LG NIEHS
A. Soni Biologist LG NIEHS
D. L. Halderman Biologist LG NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

2.4

PROFESSIONAL:

0.8

OTHER:

1.6

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are investigating the proteins used to control the level of mutations produced during the replication and maintenance of genetic information in cells. For this purpose an assay has been developed to determine the frequency and specificity of mutations produced during a single round of DNA synthesis within a biologically active DNA molecule, and the accuracy with which each of the three major classes of eucaryotic DNA polymerases (α , β , and γ) synthesize DNA has been determined, including sequence analysis of 1163 independent mutants. These analyses lead to the following conclusions:

1. DNA synthesis in vitro using purified eucaryotic DNA polymerases is not accurate enough to account for spontaneous mutation rates in vivo.
2. Each of the three animal cell DNA polymerases produces three major types of errors: base substitutions, frameshifts, and large deletions. For all three types of errors, the relative accuracies of the DNA polymerases are $\gamma \alpha \beta$.
3. Fidelity and processivity are strongly correlated.
4. Mutational specificity is influenced not only by base hydrogen bonding and stacking interactions within the DNA, but also by the protein(s) involved in the synthesis of DNA.
5. Certain single-base substitution errors may result from transient "dislocation" of a primer-template rather than from misinsertion by DNA polymerase.
6. During the course of this work, a new technique was developed for site-specific mutagenesis. It is capable of producing a desired DNA sequence alteration simply and with high efficiency, even without phenotypic selection.

These experiments should provide detailed information about the protein-nucleic acid interactions which are important in determining accuracy. The studies are being expanded to employ yeast DNA polymerases (one of which is capable of proofreading) as well as "accessory" proteins which could improve fidelity.

Research Project:

Problem: Our understanding of the mechanisms used by a cell to replicate and maintain genetic information with high accuracy is incomplete. Even less well understood are the mechanisms which generate the many mutational responses resulting from perturbation of these processes.

Objective: This project addresses two general and interrelated issues. 1. What is the contribution of each of the putative replication and/or repair proteins to accuracy? 2. At the level of protein-nucleic acid interactions, what mechanisms operate to achieve this accuracy?

Scientific Justification and Experimental Approach: The current gaps in our knowledge of fidelity determinants stem in part from the lack of a system to address questions of frequency and specificity in a manner which will allow a determination of the exact nature of these rare events and at the same time permit analysis of mechanisms at the level of protein-nucleic acid interactions. The proposed experiments are intended to provide such information for the crucial enzymes involved in the synthesis of DNA. Such information is necessary for understanding mutagenesis at the molecular level. A determination of the cellular mechanisms for achieving the accurate production and maintenance of genetic information is essential to understanding several fundamental biological processes.

Highly purified DNA replication and repair proteins are used to synthesize a DNA strand in vitro under defined reaction conditions, using as a template the single-stranded circular DNA from bacteriophage M13mp2. The DNA sequence which is the target for mutagenesis can be chosen to analyze either specific errors or a broad spectrum of errors (including base substitutions, frame-shifts, deletions or duplications). The newly synthesized complementary strand, containing the mutation to be analyzed, is expressed by transfection of the product of the in vitro reaction into competent *E. coli* cells. Mutants are selected on the basis of plaque color and are analyzed by DNA sequencing.

Recent Accomplishments: The M13mp2 forward mutational assay has been used to establish the accuracy of several representatives of each of the three major classes of animal cell DNA polymerases (α , β and γ). These enzymes have been analyzed first because they are of eukaryotic origin. In addition, they lack 3'→5' exonuclease proofreading activity, making interpretation of mechanisms less complicated. The mutation frequency analyses demonstrate that DNA synthesis in vitro using purified eucaryotic DNA polymerases is not accurate enough to account for spontaneous mutations rates in vivo. Other factors must be involved. The most likely possibilities include improvement of selectivity by polymerase accessory proteins and error correction by proofreading during replication or by mismatch correction after replication.

The DNA sequence analyses show that each of the three animal cell DNA polymerases produce three major types of errors: base substitutions, frameshifts, and large deletions. For all three types of errors, the relative accuracies of the DNA polymerases are $\gamma > \alpha > \beta$. Thus the putative replicative DNA polymerases (γ for the mitochondrial genomes, α for the nuclear genome) are more accurate than

the putative short-gap repair polymerase β . The order of accuracy provides a strong correlation between fidelity and processivity of DNA synthesis in vitro. Thus, the most accurate polymerase (γ) adds the most nucleotides to a growing chain per each association with the primer-template DNA, while the least accurate enzyme (β) dissociates between each base addition. This implies that the same interactions between a DNA polymerase and primer-template that are responsible for increased accuracy are also responsible for the enzyme remaining bound during cycles of dNTP substrate binding, phosphodiester bond formation and translocation along the template.

Two other conclusions are provided by the sequence analyses. Clearly, mutational specificity is influenced not only by base hydrogen bonding and stacking interactions within the DNA itself, but also by the protein(s) involved in the synthesis of DNA. Finally, certain single base-substitution errors, and possibly some frameshifts and deletions as well, may result from transient "dislocation" of a primer-template rather than from misinsertion by DNA polymerase.

During the course of this work a new procedure for changing the primary DNA sequence of the mutational target (and ultimately useful for restructuring proteins produced by any cloned gene) was developed. Several single-base substitution mutations were introduced into the lacZ_α gene in M13mp2, at 40-60% efficiency, in a rapid procedure requiring only transfection of the unfractionated products of standard in vitro mutagenesis reactions. Two simple additional treatments of the DNA, before transfection, produce a site-specific mutation frequency approaching 100%. The approach is applicable to phenotypically silent mutations in addition to those that can be selected. The high efficiency, 10-fold greater than that observed using current methods without enrichment procedures, is obtained by using a DNA template containing several uracil residues in place of thymine. This template has normal coding potential for the in vitro reactions typical of site-directed mutagenesis protocols but is not biologically active upon transfection into a wild-type (i.e., ung⁺) Escherichia coli host cell. Expression of the desired change, present in the newly synthesized non-uracil-containing covalently closed circular complementary strand, is thus strongly favored. The procedure has been applied to mutations introduced via both oligonucleotides and error-prone polymerization. In addition to its utility in changing DNA sequences, this approach can potentially be used to examine the biological consequences of specific lesions placed at defined positions within a gene.

Plans for Future:

Two categories of experiments will be pursued in the next two to three years, in order to directly address the two questions posed in "Objectives". The first is to continue to utilize the forward-mutation assay to assess the frequency and specificity of mutations produced in reactions using defined protein components. Emphasis will continue to be on DNA polymerases for at least one more year, since the new system now in use is powerful and too recent to have been fully exploited. The role of accessory proteins in improving accuracy will also be examined. The expectation is that such accessory proteins will affect the DNA structure (DNA binding proteins), polymerase processivity (holoenzyme subunits),

proofreading (3'→5' exonucleases) and/or perhaps substrate (dNTP) binding affinity. For these experiments we are currently emphasizing the analysis of yeast DNA polymerases and seven accessory proteins being purified by Akio Sugino. Preliminary results with prokaryotic DNA polymerases containing associated 3'→5' proofreading exonuclease activities suggest that the assay will be useful in determining the role of proofreading by yeast DNA polymerase II in correcting various types of errors. If successful, this will be the first demonstration that proofreading operates in a eucaryotic cell.

The second and related set of experiments addresses questions of mechanisms, and will use very specific mutational targets, most often in reversion assays. Two tests are currently in progress. The first is to determine the effect on frameshift mutation frequency of directly altering processivity. The second is to directly test a novel slippage and realignment model for base substitution mutations observed at a DNA polymerase-β hotspot in M13mp2.

C. Publications of Past 18 Months:

Kunkel, T.A., Loeb, L.A., and Goodman, M.F.: On the fidelity of DNA replication. The accuracy of T4 DNA polymerases in copying φX174 DNA in vitro. J. Biol. Chem. 259: 1539-1545, 1984.

Das, S.K., Kunkel, T.A. and Loeb, L.A.: Effects of altered nucleotide concentrations on the fidelity of DNA replication. In: Genetic Consequences of Nucleotide Pool Imbalances, F. J. de Serres, Ed., p. 117-126, Plenum Press, NY 1985.

Kunkel, T.A.: The mutational specificity of DNA polymerase-β during in vitro DNA synthesis. Production of frameshift, base substitution and deletion mutations. J. Biol. Chem. 260: 5787-5796.

Kunkel, T.A.: Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. U.S.A. 82: 488-492, 1985 (and patent pending).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 60146-03 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mutagenic Consequences of Defined Lesions in DNA		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div style="width: 30%;">PI: T. A. Kunkel</div> <div style="width: 30%;">Senior Staff Fellow</div> <div style="width: 30%;">LG NIEHS</div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 30%;">Others: L. Frederico</div> <div style="width: 30%;">Guest Worker</div> <div style="width: 30%;">LG NIEHS</div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Genetics		
SECTION Mutagenesis Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 0.2	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div style="width: 30%;"> <input type="checkbox"/> (b) Human tissues </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The mutagenic consequences of introducing defined lesions into DNA are being examined in a mutational system capable of detecting a wide spectrum of mutational events, both at and some distance away from the actual site of damage. The system has first been applied to depurination, the loss of a purine base from DNA. This is a frequent spontaneous lesion as well as a common intermediate in the repair of many other types of DNA damage. Depurination is highly mutagenic, as determined by transfecting depurinated M13mp2 DNA into SOS-induced competent <i>E. coli</i> cells. DNA sequence analysis of 211 mutants demonstrates that most mutations are base substitutions reflecting insertion of dAMP opposite the non-coding abasic site and resulting in characteristic transversions. Such transversions are frequently observed <u>in vivo</u> for several damaging agents which produce bulky DNA adducts, suggesting that such adducts may produce mutations either through an abasic site intermediate, or by simply precluding template base hydrogen bonding.</p> <p>The second defined lesion is a uracil residue in DNA, resulting from the heat-induced deamination of cytosine. This is suggested to be the second most frequent spontaneous lesion in DNA, representing a significant challenge to a cell at 37°C. For these studies, the first <u>in vitro</u> assay for reversion of a proline codon (CCC) has been developed. The assay is both highly specific and very sensitive. Using this and the forward-mutation assay, the rate constant of C deamination has been determined at physiological pH and salt concentrations and 37°C. The sequence specificity of C deamination has just come under examination. Eventually, the contribution of cross-strand protonation by various adducts in double-stranded DNA, leading to C deamination, will be investigated.</p>		

Research Project:

Problem: Defining the mutagenic potential of DNA-damaging agents is complicated by the fact that most agents produce a spectrum of different lesions at multiple positions and at varying frequencies and because several mutational outcomes are possible.

Objective: To define exact mutational endpoints for specific lesions in DNA. The first lesion examined is the abasic site, resulting from depurination, the loss of a purine base from the DNA. The second is a uracil residue, resulting from heat-induced deamination of cytosine.

Scientific Justification and Experimental Approach: These studies are intended to provide detailed information on the exact mutagenic potential of specific lesions in DNA. This information is critical to our understanding of the risk posed by various DNA-damaging agents. The abasic site is the first lesion to be examined for two reasons. It is by definition a non-coding lesion, and it has been estimated to occur at high frequency both spontaneously and as a common intermediate in the repair of many other DNA lesions. The second lesion to be examined is a uracil residue, resulting from the deamination of cytosine. This potentially highly mutagenic event is estimated to occur spontaneously in vivo at high frequency, and this frequency may be increased upon exposure to certain agents.

Mutagenicity is examined using the single-stranded bacteriophage M13mp2, which contains the α -peptide coding region of the lacZ gene of E. coli. Mutants obtained from a transfection assay using normal or damaged DNA are scored as plaques having an altered color phenotype when plated on the appropriate indicator plates. The exact nature of the mutational events is then determined by chain-terminator DNA sequence analysis. This system allows the exact determination of many classes of mutational events, including base substitutions, frameshifts, deletions, and duplications.

Recent Accomplishments: The mutagenic consequences of damage to DNA produced by low pH and high temperature (primarily depurination) have been determined in a forward-mutation system. Transfection of depurinated single-stranded M13mp2 DNA into competent cells results in a 15-fold increase in the frequency of mutant (light blue or colorless) plaques compared to a non-depurinated DNA control. Mutagenicity is proportional to the number of lethal sites introduced into the DNA and is largely dependent on a functional error-prone repair system in the competent E. coli cells. Approximately 90% of the damage-dependent increase in mutagenicity is abolished by apurinic endonuclease or by alkali-treatment of the damaged DNA prior to transfection. Based on these observations and the rate constants for formation of the various types of lesions produced by heat/acid in DNA, it is concluded that the majority of the induced mutagenesis results from the presence of abasic sites in the DNA. DNA sequence analysis of 87 spontaneous and 124 induced mutants indicates that three types of mutational events are increased: base substitutions (31-fold), double mutations (>33-fold) and mutations arising from recombination between the M13mp2 DNA and the complementary lac information on the F' in the host cell (16-fold). There is only a

slight increase in the frequency of frameshift mutations (3.8 fold) and essentially no increase in large deletions. Approximately 80% of the base substitution mutations occur at purine positions in the viral strand, consistent with depurination being the predominant premutagenic lesion. The 2:1 ratio of G:A sites mutated is consistent with the preference for depurination of G over A. Transversion are observed for 57 of 79 (72%) induced base substitutions, with a strong preference for insertion of A opposite the putative apurinic site.

In order to study deamination mutagenesis, a specific reversion assay has been designed, using a CCC codon in the α -peptide coding sequence of M13mp2. Using this codon, which is a highly sensitive indicator for C \rightarrow T transitions resulting from deamination, the rate constant of cytosine deamination has for the first time been determined at 37⁰ and physiological pH and salt conditions. The specificity of this deamination is being examined in the forward-mutation assay at many different C residues.

Plans for Future:

The major efforts in the next year will be to examine the sequence specificity of deamination mutagenesis and to determine if some proportion of the mutagenesis associated with certain DNA damaging agents is a result of either direct deamination of cytosine or indirect deamination resulting from "cross-strand" protonation. This latter effort should yield interesting results on the structure of both normal and damaged DNA which could have implications beyond our own interests in mutational endpoints. Depending on the interests of two new members of our laboratory, the mutational consequences of single defined lesions at specific sites in the DNA can be examined.

Publications in Last 18 Months:

Kunkel, T.A.: The mutational specificity of depurination. Proc. Natl. Acad. Sci. USA 81: 1494-1498, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 60147-02 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Mechanisms of SOS-Mutagenesis in Escherichia Coli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. M. Schaaper

Visiting Associate

LG NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

0.75

PROFESSIONAL:

0.75

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to study the molecular events that lead to mutation in the bacterium E. coli after induction of the SOS-response. The error-prone type of DNA replication that is presumed to be responsible for SOS-mutagenesis will be studied in an in vitro replication system. The accuracy with which crude extracts of E. coli cells copy normal or damaged single-stranded bacteriophage M13 DNA will be used as an indicator for in vitro SOS-expression. Characterization of the components involved is important for the study of SOS-mutagenesis and for the question of the regulation of mutation rates in general. An in vivo mutational assay was developed which demonstrated a strong mutational response when depurinated M13 molecules were transfected in SOS-induced cells. To allow the in vitro analog of this system to be tested, procedures were developed for the efficient replication of M13 ssDNA by crude E. coli extracts to yield RFI DNA, and the subsequent recovery of the product DNA for transfection to yield sufficient numbers of infective centers for an analysis of mutation. Preliminary results indicate that DNA replication as it occurs in the crude extract system is extremely accurate and may approach values expected in vivo.

Research Project

Nature of the Problem: In the study of mutagenesis in *E. coli* the SOS-system plays a central role. The system is induced when damage to the DNA of the organism blocks or impedes DNA replication. The system is multifaceted and is believed to include an error-prone replication component responsible for mutagenesis on both intact and damaged DNA. By genetic analysis several genes participating in this process have been identified. However, the biochemical nature of the mutational process has remained obscure. Elucidation of this pathway is important to our understanding of induced mutation. In addition, information is likely to be gained regarding the mechanisms by which organisms can regulate mutation rates in response to changing and challenging environments.

Objectives: We want to develop an *in vitro* assay system for the presumed error-prone DNA replication in SOS-induced cells. Increased error rates for replication by extracts derived from SOS-induced cells will be used to dissect and identify the various components of this error-prone system.

Experimental Approach and Scientific Justification: We will study the accuracy of DNA replication in crude extracts derived from either normal or SOS-induced *E. coli*. The accuracy will be measured using M13mp2 DNA which allows scoring for mutations in the α -complementation region of the *lacZ* gene (plaque color assay). The conversion of M13 single-stranded to double-stranded DNA (SS \rightarrow RF) by crude extracts has been well documented and the essential components have been identified. The accuracy of such a single round in replication in a crude extract system has not been measured, but is expected to be similar to the *in vivo* value. The DNA templates to be used will be either undamaged or will contain apurinic (abasic) sites. Such sites have been shown to be useful DNA lesions because they block normal DNA synthesis but are efficiently bypassed under SOS-induced conditions, giving rise to characteristic transversion events. In parallel, *in vivo* assays will be developed in which the depurinated M13 DNA is transfected into SOS-induced calcium-treated-cells, allowing a direct comparison of *in vivo* and *in vitro* results. Once positive *in vitro* responses have been obtained, efforts will be directed at defining the nature of the step(s) leading to increased error rates. A knowledge of the factors contributing to normal accuracy will be necessary for such an evaluation. Thus, a description of baseline *E. coli* replicational fidelity in terms of the specificity of mutation, the contributions of base selection vs proofreading, and other potential modulating factors, will also be pursued.

Recent Accomplishments and Significance to Biomedical Research:

Replication/ recovery/transfection: Optimum conditions have been established for the efficient conversion of M13 SS DNA to RFI DNA by crude extracts from either normal or SOS-induced cells. Procedures were developed for the recovery of the product DNA and the subsequent transfection into calcium-treated-cells to yield sufficiently high numbers of infective centers to permit mutation analysis.

Mutation assay: A series of in vivo experiments was performed to establish the magnitude of the SOS-response when transfecting depurinated M13mp2 DNA into SOS-induced cells. Greatest sensitivity (signal to noise ratio) was observed using a mutational reversion system. For example, the background revertant frequency of a selected M13 amber mutation (reversion to wild-type) was 2×10^{-6} ; in case of depurination and transfection into SOS-induced cells, a frequency of 2×10^{-4} was obtained. This high frequency suggests efficient (10%) in vivo bypass of the apurinic sites when SOS has been previously induced.

In vitro mutagenesis: A limited number of in vitro experiments has been performed. Reversion frequencies significantly above the background frequency have not yet been obtained. It is still to be determined whether this points to a reduced efficiency of the SOS processes when acting in vitro or to a more fundamental difference between in vitro and in vivo fidelity. Further experiments will be directed at resolving this issue. More detailed insights into the specificity and fidelity components of normal DNA replication will probably be required (see next section).

Fidelity of DNA replication in crude extracts: The type and frequency of the various DNA replication errors which occur when copying M13 DNA with a crude *E. coli* extract have been determined by performing DNA synthesis under a variety of different dNTP concentrations and ratios, followed by DNA sequence analysis of the resulting mutants. The major finding thus far is the high accuracy of the SS \rightarrow RF conversion. When measuring the reversion of amber A88, all eight possible sense codons have been scored. Their combined (background) frequency is 5×10^{-6} . However, when specifically eliciting mutations at each of the three positions by pool imbalances, only the G \rightarrow T mismatch error at the first position of the codon seems to be promoted, producing a TAG \rightarrow CAG transition. The frequency for this event extrapolated to standard dNTP pools is 4×10^{-7} . This value is obtained at high concentration of the "next nucleotide", implying that, at physiological (lower) dNTP concentrations, this frequency might be even lower due to more active proofreading by the 3' \rightarrow 5' exonuclease of the pol III holoenzyme complex. In general, increased overall accuracy has been observed when lowering dNTP levels, consistent with an important role for proofreading in replicational fidelity in this system. Future efforts will be directed at a direct quantification of this effect for each individual position in this (and other) amber codon(s). Because reduced proofreading capacity in SOS-induced cells has been proposed as a mechanism by which SOS-induced mutagenesis could take place, this will be an important criterion when comparing replication by SOS⁺ and SOS⁻ extracts. The observed high fidelity of replication provides a major challenge to the experimenter. At the same time, however, it indicates the "truthfulness" of the replication taking place in the present assay system. In contrast, it has been possible to obtain high error rates by the addition of Mn⁺⁺, a well known mutagen in replication systems which make use of purified DNA polymerases. DNA sequence analysis showed that the increase in mutation frequency under these conditions is due to both transition and transversion mutations. We interpret the combined findings to indicate that the present replication/mutation system is capable of detecting replication errors when they occur and is a promising tool to study highly accurate replicational processes. The significance of this project to biomedical research is severalfold. Knowledge of the molecular mechanisms of SOS-induced mutation is quintessential for our understanding of mechanisms of mutation in general. Evidence for

SOS-like responses in organisms other than E. coli, including mammalian cells, is increasing. The accuracy of DNA replication per se is directly related to the accuracy with which organisms transfer their genetic information from generation to generation. Discovery of the mechanisms underlying SOS may place a new perspective on the question how organisms may regulate mutation rates in response to changing environments.

Plans for Future:

We will continue to pursue a full characterization of replicational fidelity in crude E. coli extracts, since the present system represents the closest approximation to in vivo conditions currently available. We will determine, where possible, the frequencies of individual mismatches, their dependence on substrate-concentrations (as a way to quantitate the contributions of base selection and proofreading), and the possible influence of allosteric effectors. A comparison of such parameters in SOS^+ and SOS^- extracts will serve as basis for delineating the nature of the error-prone components of SOS.

Publications:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 60148-02 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Error-Prone Repair in Bacteriophage T4

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. W. Drake Head LG NIEHS

Others:	F. W. Coleman	Senior Staff Fellow	LG NIEHS
	L. K. Derr	Biologist	LG NIEHS
	A. G. Morton	Biological Aid	LG NIEHS
	D. C. Nguyen	Chemist	LG NIEHS
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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

2.8

PROFESSIONAL:

2.3

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The central objective of this project is the analysis of error-prone repair in bacteriophage T4, with related explorations into other mechanisms of mutagenesis and into a novel mechanism for avoiding ultraviolet-induced killing. Most chemical and all radiation mutagenesis in T4 occur via error-prone repair and depend on the functions of the genes uvrW, uvrX and uvrY, most or all of the genes required for DNA replication, and perhaps several other genes as well. We have recently characterized temperature-sensitive alleles of the X and Y genes, alleles that differentially affect mutagenesis and inactivation. Some of the mutant X proteins are being isolated in order to determine which of their kinetic parameters correlate with mutagenesis and which with survival. A number of tests have been or will be conducted to characterize the role of the WXY system in photodynamically induced mutagenesis and killing, to determine if other T4 genes are involved in WXY action (especially genes 46, 47, 49, 58, 59 and uvrZ), to more deeply explore the role of the uvrW gene, and to characterize a newly discovered role in survival after UV irradiation for the T4 gene 32 protein (the major viral DNA-binding protein).

Research Project

Most mutagenesis induced by most chemicals and radiations in most organisms occur during the course of error-prone repair (EPR), a process operationally defined by mutants that exhibit increased sensitivities to inactivation and decreased sensitivities to mutagenesis by diverse mutagens. Analysis of the mechanism(s) of EPR has proceeded most rapidly in two microbial systems: the SOS system of *E. coli* and the WXY system of bacteriophage T4. The problem of the moment is to determine which genes are involved in this mutagenic response, and how. Ultimately, the problem is to learn how to manipulate the EPR process in ways that decrease mutagenesis but do not unduly decrease cell survival or function.

Much of our work bears on the questions of the mechanism of EPR and its evolutionary significance and grows out of recent work in this laboratory (Conkling and Drake 1984a and b) in which temperature-sensitive mutations of uvrX and uvrY were isolated and characterized. Many of these ts mutations affect the three WXY functions examined to date to very different extents, indicating that diverse WXY functions can be uncoupled mutationally and suggesting that they may be affected by different domains of the uvrX and uvrY proteins. The three functions in question are suppression of a gene 49 (Holliday resolvase) defect, UV-induced inactivation, and UV-induced mutagenesis. Out of this work emerged the hypothesis that EPR occurs in the immediate vicinity of a newly established recombinational intermediate. (The competing hypothesis, due to Radman and in vogue for nearly a decade, holds that a DNA-damage-induced protein leads to EPR by reducing polymerase fidelity; such a reduced fidelity has not been convincingly demonstrated in vitro despite many man-years of effort in several laboratories, two claims to the contrary being artefacts or irrelevancies.) More specifically, a DNA polymerase-blocking lesion (such as a dimerized pair of adjacent pyrimidines) must be bypassed ("bypass synthesis") by the polymerase for EPR to occur, and we believe that such bypasses can occur more readily within the context of a D-loop than within an ordinary replication fork.

Although error-prone repair is usually carried out by the same system that conducts recombination and recombination-like post-replication repair, no adequate tests have been reported to explore the linkage, if any, between induced mutagenesis and induced recombination. (Most mutagens acting through EPR enhance recombination as well as mutation.) Two approaches are planned to test whether the two kinds of events are correlated. First, using our collection of temperature-sensitive uvrX and uvrY mutations, we will test whether these affect UV-enhanced recombination frequencies in a manner parallel or discordant with their effects on UV mutagenesis. Second, attempts are being made to ask whether recombinants are enriched for mutants and vice versa; in a previous test studying proflavine mutagenesis (which operates independently of the EPR system), no correlation was found.

A number of T4 genes are additional candidates for involvement in ERP. These genes (46, 47, 49, 58, 59, mms and uvrZ), when bearing amber mutations, are nevertheless able to grow to a limited extent in a nonsuppressing cell, and are already known to be defective in recombination, survival following UV irradiation, or both. Techniques have now been developed so that mutagenesis can be studied in these mutants growing in nonsuppressing cells. Not only are these

studies more precisely defining which genes are involved in mutagenesis, but they may also add to our studies of the correlation between mutagenesis and recombination.

Defects in EPR, particularly those primarily affecting its mutagenic component (as contrasted, for instance, to error-free recombinational repair), produce only relatively small effects on cell or viral survival following treatment with diverse chemicals or radiations, especially compared with the large effects upon survival resulting from defects in excision or recombinational repair. We have therefore long postulated that an important, perhaps the most important, adaptive advantage of EPR was the increase in genetic variance it engendered in response to environmental hostility. A competing hypothesis would be that nobody had yet examined the appropriate source of lethality, one that would be strongly repaired by EPR. Recently, however, the Eisenstadt group has reported that isopsoralen-mediated photodynamic inactivation proceeds many-fold faster in *E. coli* bearing a mutation specifically blocking the mutagenic component of EPR. We have therefore asked whether a similarly high sensitivity is seen in a T4 uvsX mutant. The answer is no (Drake, 1985). In addition, we rechecked some published reports to the effect that a denV mutation partly or completely overcame the sensitizing effect of a uvsX mutation upon photodynamic inactivation, a result unexpected because the denV gene encodes the viral cyclobutane dimer excision system, a system that does not operate at all on other kinds of damage. No such V-X functional interaction was seen in our tests.

Since the ts mutations of uvsX and uvsY that we recently isolated have differential effects upon UV killing and mutability, we have initiated a project to isolate and characterize the mutant proteins from these strains, hoping to find kinetic differences that will correlate specifically with their effects upon killing versus mutagenesis. The uvsX protein displays a DNA-dependent ATPase activity, promotes several strand-exchange reactions, and binds to other T4 proteins such as those encoded by genes uvsY and 32, the latter being the major viral DNA-binding protein. Since the uvsY protein has, as yet, no kinetically defined characteristics, we also plan to look for such activities. These studies, which are in their early phases, are being conducted in collaboration with the Sugino group.

Because of the interesting results obtained by studying the characteristics of ts mutations of uvsX and uvsY, we have initiated a project to collect and study ts mutants of uvsW. W mutants may be obtained in the first place as suppressors of mutations in gene 59, a gene which is almost surely itself a component of the WXY system. Since W mutants are sensitive to hydroxyurea, their isolation should be straightforward. They will then be tested for sensitivity to UV-induced killing and mutagenesis, and for effects upon recombination, at both high and low temperatures. Ultimately, the more interesting of these mutants will be selected for biochemical analysis.

The scoring of uvsW, X and Y mutations on the basis of their UV-sensitivity and complementation patterns has long been sufficiently tedious to seriously impede their analysis. We have recently assembled (from friends) and characterized a set of plasmids carrying the wild-type alleles of each of these genes, singly, and find them to be most helpful.

Several years ago we noticed accidentally, during the course of strain constructions, that an amber mutation of gene 32 displayed increased UV sensitivity. Classical studies implicated this gene in both excision and WXY repair processes, although the evidence is uncomfortably indirect in both cases. Upon constructing a T4 strain carrying defects in both the V and the WXY system, as well as the gene 32 mutation, we observed that the triple mutant was substantially more UV-sensitive than the double (V-X or V-Y) mutant. This remarkable result defines a totally new process for the avoidance of UV-induced lethality. The defect is seen when the amber mutant is grown under permissive conditions (that is, in a suppressor host cell), and is of equal magnitude regardless of what (allowable) amino acid is inserted, suggesting that the component of the infection that reduces survival is the amber polypeptide fragment rather than the missense protein. UV mutability is completely normal in this gene 32 mutant, further complicating older claims that gene 32 is involved in WXY repair.

Plans for Future:

Work outlined above to pursue the correlation between recombination and mutagenesis, to characterize biochemically the mutant uvsX and uvsY proteins, to obtain and characterize temperature-sensitive uvsW mutants, and to obtain a deeper insight into how the gene-32 effect on UV killing occurs, will totally occupy this group for at least two years. The initial studies on gene 32 are now being prepared for publication.

Publications in Past 18 Months:

Coleman, F.E., and Sugino, A.: Purification of a DNA primase activity from the yeast Saccharomyces cerevisiae: primase can be separated from DNA polymerase I. J. Biol. Chem. 260: (in press).

Crow, J.F., Abrahamson, S., Denniston, C., Hoel, D.G., Huberman, E., Magee, P.N., Nebert, D.W., Roderick, T., Shaw, M.W., Sherman, F., Simmon, V.F., Sutton, H.E., Wolff, S., and Drake, J.W.: Identifying and Estimating the Genetic Impact of Chemical Mutagens. Washington, National Academy Press, 1983.

Drake, J.W., and Ripley, L.S.: The analysis of mutation in bacteriophage T4: delights, dilemmas and disasters. In Mathews, K. et al. (Ed.): Bacteriophage T4. Washington, Am. Soc. Microbiol., 1983, pp. 312-320.

Drake, J.W., Glickman, B.W., and Ripley, L.S.: Updating the theory of mutation: de mutatis mutationum mutandis. Am. Scientist 71: 621-630, 1983.

Drake, J.W.: Viewing the complexities of mutagenesis from an evolutionary perspective. In de Serres, F.J. et al. (Ed.): Genetic Consequences of Nucleotide Pool Imbalance. New York, Plenum Press, 1985, pp. 335-338.

Sugino, A. and Drake, J.W.: Modulation of mutation rates in bacteriophage T4 by a base-pair change a dozen nucleotides removed. J. Mol. Biol. 176: 239-249, 1984.

Conkling, M.A., and Drake, J.W.: Isolation and characterization of conditional alleles of bacteriophage T4 gene uvrX and uvrY. Genetics. 107: 505-523, 1984.

Conkling, M.A., and Drake, J.W.: Thermal rescue of UV-irradiated bacteriophage T4 and biphasic mode of expression of the WXY system. Genetics. 107: 525-536, 1984.

Ripley, L.S., and Drake, J.W.: Bacteriophage T4 particles are refractory to bisulfite mutagenesis. Mutation Res. 129: 149-152, 1984.

Drake, J.W.: Photodynamic inactivation and mutagenesis by angelicin (isopsoralen) or thiopyronin (methylene red) in wild-type and repair-deficient strains of bacteriophage T4. J. Bacteriol. 162: (in press), 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 60150-02 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Gene Induction by Alkylation Treatments in E. coli		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. R. Volkert	Sen. Staff Fellow LG NIEHS
Others:	D. C. Nguyen	Chemist LG NIEHS
	A. G. Morton	Biological Aid LG NIEHS
	E. C. Pollard	Guest Worker LG NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Genetics		
SECTION Mutagenesis Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 0.8	OTHER: 1.75
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>When <u>E. coli</u> cells are treated with sublethal levels of alkylating agents, they become <u>more</u> resistant to the lethal and mutagenic effects of subsequent high-level treatments with alkylating agents. This increased resistance has been called the adaptive response to alkylating agents. It is the result of the induction of genes that increase the cell's capacity to repair alkylation lesions in DNA. We have initiated a genetic study designed to learn what genes are induced by alkylation treatments, how they are regulated and what are the functions of their products. To date five genes or operons have been identified that are induced by alkylation treatments. They were identified as fusions of the <u>lac</u> operon to promoters that caused increased β-galactosidase activity upon alkylation treatment. Of these <u>lac</u> fusions, two are linked to promoters of genes known to be involved in the repair of alkylation damage. The <u>aidA</u> (alkylation inducible) gene codes for the <u>alkA</u> gene product and <u>aidD</u> is a fusion to the promoter of the <u>ada/alkB</u> operon. <u>aidB</u>, <u>aidC</u> and <u>aidI</u> represent fusions to genes that had not been previously identified. Studies of the regulation of these <u>aid</u> genes shows that <u>aidA</u>, <u>aidB</u> and <u>aidD</u> fall into one regulatory group and are controlled by the <u>ada</u> gene, a regulatory element needed for normal expression of the adaptive response. <u>aidC</u> and <u>aidI</u> each appear to be regulated separately and are not under <u>ada</u> control. Moreover, <u>aidC</u> is induced by MNNG but not by MMS, whereas <u>aidI</u> is induced by MMS but not by MNNG. Therefore, these two genes must respond to different induction signals.</p>		

Research Project:

Problem: When *E. coli* cells are treated with low levels of alkylating agents they become more resistant to the lethal and mutagenic effects of subsequent high-level challenge treatments with alkylating agents. This induced resistance has been termed the adaptive response, and is due to the induction of genes whose products act to repair specific alkylated nucleotides and phosphotriesters. The two major activities that are increased during adaptation are glycosylases and methyltransferases. The glycosylase activities, which are encoded by the *alkA* gene, cleave the glycosylic bond between the base and the sugar of the DNA backbone, thus removing several alkylated bases (N^3 -methyladenine, N^3 -methylguanine, N^7 -methylguanine, O^2 -methylcytosine, and O^2 -methylthymine). Methyltransferases act to remove alkyl groups from several alkylated bases in DNA, restoring the base to its natural unmethylated state. Methyltransferases act specifically to repair O^6 -methylguanine, O^4 -methylthymine and methylated phosphotriesters.

The full scope of the adaptive response is presently not known, nor is it clear how the inducible components of the adaptive response are regulated at the molecular level. Our goals are to identify the genes induced upon alkylation treatment, to develop an understanding of their regulation, and to learn what are the products of the genes and what are their roles in DNA repair or cellular recovery from alkylation treatment.

Experimental Approach: Our approach to the adaptive response has been to construct random operon fusions of the *lac* operon to *E. coli* genes using the phage Mu-d1(Ap^R *lac*). This phage contains the *lac* operon but partly deleted such that the β -galactosidase gene (*lacZ*) has no promoter. β -galactosidase activity can be expressed only when this phage has been inserted into a gene in the proper orientation such that transcription begins at the promoter of the target gene and proceeds into the *lacZ* gene of the phage. In addition to allowing β -galactosidase activity to be expressed, this type of insertion places the *lacZ* under whatever regulatory control is exerted on the external promoter. Thus, by selecting for fusion-containing strains that express β -galactosidase only after treatment with alkylating agents, it is possible to select for fusions to genes that are normally induced in response to alkylation treatment. In addition to providing a way to select for fusions to alkylation-inducible genes regardless of gene function, the resultant fusion provides a convenient and useful tool with which to measure gene expression simply by measuring β -galactosidase activity. Mutant phenotypes often result whenever the sequence of the structural portion of the target gene is interrupted by the phage genome. Thus, the function of the target gene can often be deduced from phenotypic characterization of the fusion mutant.

These fusions also provide convenient tools for molecular cloning of the promoter/operator regions, thus allowing one to examine the regulatory regions at the level of DNA sequence. Such clones are also effective probes for subsequent identification of clones of the wild-type alleles from genomic libraries.

Based on these features, operon fusion technology provides a powerful tool for the study of genes and their regulation. Operon fusions are especially useful when genes are known only in terms of their regulatory responses.

Recent Accomplishments: To date we have isolated a total of 14 lac fusion strains representing at least 5 different genetic loci that are induced by alkylation treatments. These fusions have been called aid (alkylation inducible) mutations. Included among the 5 loci are several fusions to the alkA promoter and one to the ada promoter. The recovery of these two mutants has shown that the isolation procedure is yielding the expected fusions as well as fusions to previously unidentified genes. Use of the alkA and ada fusions has allowed us to quantify the induction of these genes after alkylation treatment and to measure their responses to a number of different DNA-damaging agents. These fusions also provided us with controls to compare with fusions to newly identified genes.

Three separate regulatory mechanisms control aidA(alkA), aidB, aidC, aidD(ada) and aidI. aidA, aidB and aidD are controlled by ada, the regulatory gene for the adaptive response, and are induced by a variety of methylating agents. aidC and aidI are not affected by ada mutations and are therefore under separate regulatory control. Moreover, these two genes show different responses to different methylating agents. For example, aidC is induced by N-methyl-N-nitro-N-nitrosoguanidine (MNNG) but not by methyl methanesulfonate (MMS). aidI shows the opposite response to these two agents. Thus, aidC and aidI are regulated independently of one another. Based on these results, the cellular responses elicited by alkylating agents differ depending upon the alkylating agent used. Such differences in gene induction are likely to be linked to the mode of alkylation by an agent, the types of lesions it produces, or its metabolism by the cell.

A number of regulatory mutants have been isolated that exhibit altered regulation of an ada-controlled fusion. Many of these show constitutive expression of β -galactosidase. All constitutive mutants are highly unstable, segregating revertants at a frequency of 10-50%. These mutations can be stabilized by introducing a recA mutation that blocks recombination. This result is indicative of tandem duplications. The idea that tandem duplications can result in constitutive expression of an aid::lac operon fusion is consistent with the hypothesis that these genes are controlled by a positive regulatory element. Tandem duplication of a positive regulatory element would result in an increased gene copy number and an increased basal level of the regulatory protein. This in turn would cause activation of the genes under its control. Experiments are currently in progress to establish conclusively that constitutive expression of ada-controlled genes is due to tandem duplication of a positive regulatory element.

Phenotypic studies have shown that aidA and aidD fusions reduce cellular survival after alkylation treatment, indicating that these genes are required for repair or recovery. Several aidB fusions actually increase survival from MNNG treatment, suggesting that this gene may be involved in the conversion of MNNG to the proximal mutagen dimethylnitrosamine. aidC and aidI fusions have little or no effect on cell survival.

To date, we have identified several alkylation-inducible genes identified by insertion mutagenesis and have used the fusions to learn about their regulation.

Plans for Future:

This work is anticipated to take two major directions over the next few years. One is aimed at learning about the regulation of the various aid genes. The other is to learn more about the products of the genes and their roles in DNA repair.

Two projects are currently underway that address questions about the regulation of the aid genes. The first is the isolation of regulatory mutants that affect the expression of aid genes. The second is the molecular cloning of the fusions along with their operator/promoter regions, in order to identify and obtain DNA sequence information about their regulatory regions. These clones will also provide probes useful in the isolation of wild-type aid genes, which will aid in the identification of their gene products.

Publications:

Volkert, M.R. and Nguyen, D.C.: Induction of specific Escherichia coli genes by sublethal treatments with alkylating agents. Proc. Natl. Acad. Sci. USA 81: 4110-4114, 1984.

Volkert, M.R., Nguyen, D.C. and Beard, K.C.: Gene induction by alkylation treatment: The AID responses of Escherichia coli. Genetics (in press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 60151-02 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of the recF Gene in DNA Repair

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. R. Volkert Senior Staff Fellow LG NIEHS

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Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triange Park, North Carolina 27709

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The recF gene is required for two basic genetic processes, genetic recombination and DNA repair. The major question about recF centers on whether it functions in a regulatory capacity controlling these processes or whether it has an enzymatic activity needed to carry out these reactions. Our studies of suppression of recF by several recA mutations suggests that the recF gene product functions primarily by modulating recA activity. It appears that two recF-dependent changes in recA activity normally occur in response to DNA damage. The first results in activation of the recA-dependent proteolytic activity required for induction of the SOS pathways of DNA repair. The second allows recA to carry out the RecF pathway of recombination and recombinational DNA repair. These two changes in recA activity are separable by mutation since the srfA mutation of the recA gene restores only RecF recombination without concomitant restoration of SOS induction in recF mutant strains.

Research Project

Problem: The recF gene of E. coli is required for a UV-inducible pathway of recombinational DNA repair. This pathway can also carry out general genetic recombination in the appropriate genetic background. In addition, recF is required for expression of the inducible SOS pathways of DNA repair after UV treatment. The induction of the SOS response is a two-step process. The first step is the activation of the RecA protein, the second is the cleavage of LexA and other repressors of SOS genes. This proteolytic cleavage results from the interaction of the activated RecA protein with the repressors and causes the induction of the genes they control.

The understanding of how these two gene products function in recombination and repair will aid our understanding of the cellular responses to DNA damage. This work will provide information about how such repair and recovery processes are regulated and how they function at the molecular level.

Experimental Approach and Accomplishments: Our first approach to the study of the role of recF was to introduce the recA441 mutation into a recF mutant strain. This recA mutant allele produces an altered RecA protein which can be activated to cause expression of the SOS response in the absence of DNA damage, simply by incubating the cells at 42°C. Under these incubation conditions, recA441 suppresses many of the recF defects and circumvents the need for recF in SOS induction. This suggests that the recF gene may function to modulate recA activity, catalyzing the conversion of recA from its inactive to its activated form after UV damage. Experiments designed to determine if the RecF pathway of recombination was also restored under these conditions generated equivocal results.

In order to learn more about the interaction of recF with recA, we have isolated suppressor mutants that restore UV resistance to recF mutant strains. One class of recF suppressors, which we call srfA, map at recA and restore the recF pathway of recombination and recombinational repair. Molecular cloning of the srfA mutation shows that it lies between two BamHI sites that flank recA. DNA sequencing of these clones will establish conclusively whether the genetic changes lie within the recA coding sequence and the exact nature of the genetic change.

Since srfA mutations restore only RecF recombination to recF mutants and do not restore SOS induction capabilities, the mechanism of suppression of recF by srfA differs from that of recA441. However, both types of suppression are a result of alterations in recA activities. Since both types of recA changes restore different components of recF-dependent process, we have proposed that in wild-type cells recF normally functions to modulate recA activities, and that two changes in recA activity occur after UV damage. The first change is conversion to its proteolytically active form, resulting in SOS induction. The second change allows recA to carry out the RecF type of recombination. Although these changes normally occur simultaneously after DNA damage, they are separable by mutation, since srfA mutations restore only the recombinational activity to recF mutants.

Plans for Future:

Our current efforts on this topic are designed to quantify the effects of srfA mutations on SOS induction in recF mutant strains, to determine which aspects of the recF phenotype are restored by srfA, and to determine what changes in recA allow it to suppress recF.

Publications:

Volkert, M.R. and Hartke, M.A.: Suppression of *Escherichia coli* recF mutations by recA-linked srfA mutations. J. Bacteriol. 157: 498-506, 1984.

Volkert, M.R., Margossian, L.J., and Clark, A.J.: Two component suppression of recF143 by recA441 in Escherichia coli K-12. J. Bacteriol. 160: 702-705, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61005-06 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthesis and function of RNA Polymerase II in *Drosophila melanogaster*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. A. Voelker Research Geneticist LG, NIEHS

Others: L. L. Searles Staff Fellow LG, NIEHS

S. S. Huang Bio. Lab. Tech. LG, NIEHS

G. B. Wisely Bio. Lab. Tech. LG, NIEHS

COOPERATING UNITS (if any)

Dr. Arno Greenleaf, Department of Biochemistry
Duke University, Durham, North Carolina

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study was initiated to genetically analyze the biosynthesis and function of the components of the RNA polymerase II transcription complex in *Drosophila melanogaster*. RNA polymerase II is a heteromultimer consisting of approximately ten different subunits, each of which is presumably specified by a different locus. The number of associated transcription factors (which are not structurally a part of RNA polymerase II) is unknown, but evidence for their existence has been found in other systems. To date only the genetic locus that which specifies α -amanitin-resistance to RNA polymerase II has been identified. That locus has now been cloned as recombinant DNA molecules and was found to encode the 215,000 dalton subunit. The genetic control of the biosynthesis of that subunit is being analyzed at the molecular level by analyzing a number of revertants of the P-element induced mutant that was used to clone the DNA sequences of the region.

PROJECT DESCRIPTION

PROBLEM: RNA polymerase II, which is a heteromultimer consisting of about ten different subunits, is the enzyme complex that functions in the transcription of messenger RNAs in eukaryotic organisms. This study was initiated to genetically analyze the genes involved in the biosynthesis and functions of this enzyme complex.

OBJECTIVES: The objectives are to identify the genes encoding the various subunits of the complex and characterize them at the molecular level. Understanding the genetic control of the coordinate biosynthesis of the complex is a long-range goal.

METHODS EMPLOYED: The basic approach of this study is to utilize genetic techniques of analysis to identify mutants of RNA polymerase II that can be subsequently analyzed at the biochemical and molecular levels. The one locus identified was discovered by screening for α -amanitin-resistance. The locus was mapped by standard recombinational and deletion mapping techniques. It was further characterized by induction and genetic/molecular characterization of additional mutants at the locus.

MAJOR FINDINGS AND PROPOSED COURSE: A locus that confers α -amanitin resistance to RNA polymerase II and encodes the 215,000 dalton subunit has been identified. The locus is lethal-mutable. Different alleles at the locus affect male fertility and act as enhancers of alleles at the other loci such as Ubx and ct. The C4 locus has been molecularly cloned by identifying and recovering mutants at the locus that were caused by hybrid-dysgenesis-induced insertion of the P-element transposon. One of these P-element-induced mutants has been found to be genetically very unstable in dysgenic flies. It reverts with frequencies of 2.5 to 6.5 percent by excision of the P-element, with many of the revertants resulting from imprecise excision. The consequences of these imprecise excisions on function were analyzed. In addition, the RpII215 region was analyzed to identify all genetic complementation groups and all transcripts produced in order to correlate messenger RNA's with genetic complementation groups. More transcripts from the region have been detected than genetic complementation groups and some complementation groups have been identified with particular messages. Although negative evidence is not compelling, there is no genetic evidence that would suggest that any of the messages in the RpII215 region encode any subunit of RNA polymerase II other than the 215,000 dalton subunit. The completion of this genetic/molecular analysis completes the involvement in this project. Further work on the problem will continue in the laboratory of Dr. Arno Greenleaf in whose laboratory the project was initiated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: RNA polymerase II is an important enzyme in eukaryotic gene regulation and development. The details of the role of this enzyme are very poorly understood, and the prospects of a comprehensive genetic and biochemical analysis of such a complex enzyme with mammals are very bleak. Therefore, we have chosen to approach the problem with *Drosophila*, a well-defined eukaryotic genetic system which allows a powerful combination of genetics and biochemistry. Moreover, it appears that

the structure and function of Drosophila RNA polymerase II is very similar to that in humans and other mammals; thus knowledge obtained in Drosophila can probably be transferred to and utilized in the human situation with relatively little modification.

Before we can understand the risks of environmental mutagens and carcinogens, we must know how they affect the fundamental processes of cell growth and development. The effort of this study is to determine the role of RNA polymerase II in normal cell function. When that knowledge is available we can begin to assess how the various environmental insults impinge on normal cellular function.

PUBLICATIONS

Voelker, R.A., Greenleaf, A.L., Gyurkories, H., Wisely, G.B., Huang, S.-M., and Searles, L.L.: Frequent imprecise excision among reversions of a P element-caused lethal mutation in Drosophila. Genetics 107: 279-294, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61011-05 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Organization and Regulation of Gene Function in D. melanogaster

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. H. Judd Research Geneticist LG, NIEHS

Others: Patricia S. Davis Chemist LG, NIEHS

Deborah A. Adams P Appointment LG, NIEHS

Katherine M. Peterson Q Appointment LG, NIEHS

COOPERATING UNITS (if any)

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SECTION

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INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Study continued under Project Number Z01 ES 65036-01 LG entitled "Gene Organization and Regulation in D. melanogaster".

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61018-05 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

DNA Sequence Variation in the Alcohol Dehydrogenase Gene Region of *Drosophila*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Charles H. Langley	Research Geneticist	LG, NIEHS
	Charles F. Aquadro	Senior Staff Fellow	LG, NIEHS

Others:	Carol Reeb	Bio. Lab. Tech.	LG, NIEHS
	William F. Quattlebaum	Bio. Lab. Tech.	LG, NIEHS
	Kathy Sykes	Q Appointment	LG, NIEHS
	Stephen Schaeffer	Summer Appointment	LG, NIEHS

COOPERATING UNITS (if any)

Dr. C. Laurie-Ahlberg and Dr. H. Tachida, North Carolina State University
 Raleigh, North Carolina
 Dr. T. Mukai, Kyushu University, Japan

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

1.0

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Variation in the DNA restriction map in the *Adh* region (alcohol dehydrogenase locus) of chromosome II of *Drosophila melanogaster* from natural populations was examined. Two and a half percent of the nucleotides are polymorphic in the 12 kilobase region examined. In addition, insertions and deletions are common. Insertions of over 200 nucleotides are transposable elements. The frequency distribution within *Drosophila melanogaster* and among related species suggests that such variants are deleterious mutants. Comparisons of *Adh* gene activity among inserted and noninserted sequences support this view for some variants. Selection for a limited element copy number appears to be the most likely general explanation for their deleterious behavior. Evidence is found for preferential insertion of transposable elements into sequences implicated as being important in gene regulation. The main element involved has been sequenced and shown to be a new member of the F-like family. Two levels of *Adh* activity (high and low) commonly segregating in natural populations appear due to one or more nucleotide substitutions within the transcript that in some way alter *Adh* expression (possibly by affecting mRNA stability, processing or translation). Examination of *Adh* region sequences in 59 lines of *Drosophila* that have collected mutations for 300 generations ($59 \times 300 \times 2 = 35,400$ allele generations) shows a significant increase in *Adh* activity variation but no structural gene or flanking sequence changes. *Adh* region variation has also been examined by restriction mapping and DNA sequencing in two related species, *D. simulans* and *D. pseudoobscura*. It is clear that the target for mutations of significant effect is substantially larger than the coding sequence for the gene product.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: Our knowledge of the quantity and significance of genetic variation is largely limited to transcribed regions of the genome. However, these regions represent only an extremely small portion of the eukaryotic genome. Recent technical advances in molecular biology and DNA technology have made it possible to examine in detail the previously inaccessible noncoding genetic material. Studies have revealed a remarkable diversity of variation can exist in these noncoding regions, yet the significance of much of it remains unknown. Also, we know little of the role these sequences play in proper gene expression. What is the target for mutations of significant effect?

OBJECTIVES: We have focused on an intensively studied genetic locus in Drosophila melanogaster, that encoding the enzyme alcohol dehydrogenase (Adh). Our objectives have been to discover 1) What levels of DNA sequence variation normally occur in natural populations (i.e., insertions, deletions, nucleotide substitutions, rearrangements, transposable elements, etc.); 2) Where, relative to structural genes or other regions of defined function, these alterations occur; 3) What effects, if any, these alterations have on the expression of the genes in these areas of the genome?

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: We have chosen to study D. melanogaster from natural populations for several reasons. The genome size of Drosophila is more than an order of magnitude smaller than that of mammals, making the studies significantly more feasible technically. There also exists a tremendous wealth of genetic and molecular data for the Drosophila genome (and the Adh gene in particular) on which to draw as a resource. In addition, the documentation of, for example, the effect of insertions of transposable elements on the expression of adjacent genes is extremely difficult without detailed genetic mapping studies of the gene activity effects. Such studies are much more feasible in Drosophila than in mammals. The study of naturally occurring DNA sequence variation is also central to the mission of the Institute because it provides information on the background levels of variation, information vital to the assessment of the significance of mutagen exposure. The results also contribute significantly to determining the "target" for mutagenesis and the types of variation that can and cannot be tolerated.

Forty-nine genetically isolated second chromosomes of D. melanogaster were reared and nuclear DNA isolated. Restriction endonuclease site maps were constructed for a 12 kilobase region including the Adh structural gene. These maps were then compared for evidence of nucleotide substitutions and insertion/deletion variation. Insertions and deletions were cloned and characterized by restriction mapping, sequencing and comparison of sequence homology with previously described transposable elements. Adh gene expression was measured in adults by assaying ADH enzymatic activity. Some lines were also examined at the larval stage and analyzed for altered levels of Adh-specific messenger RNA. An additional 60 lines of D. melanogaster representing additional natural populations and variants were also examined for restriction map variation in the Adh region.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: We have mapped and scored 31 restriction sites, eight of which were polymorphic. From the frequency of site variation in these populations we estimate that 1 in 50 nucleotides vary and that between any two randomly chosen chromosomes, 1 in 200 nucleotide sites will differ. All lines have also been scored for their ADH allozyme (Fast vs. Slow) by starch gel electrophoresis. This difference in mobility is due to a Thr vs. a Lys at amino acid position 192 and reflects a single nucleotide substitution.

More remarkable than the extent of nucleotide substitution variation is the diversity of sequence length variation. Eighty percent of the lines have at least one insertion or deletion relative to the most common restriction map. These insertions/deletions range in size from 27 to over 9000 bp in length. This length variation is distributed over most of the 12 kb region with some notable exceptions. Clustering of length variation is apparent approximately 1.5 kb 3' to Adh. Four sizes of inserts, ranging from 340 bp to over 9 kb, were observed in a 340 bp region in eight of the 49 lines (one insert, "b", occurs in five different lines). No insertions/deletions were observed in Adh coding sequence or in the immediate 3' untranscribed region. Three small (27 to 38 bp) insertions/deletions were, however, located in sequence corresponding to the 5' untranslated portion of the adult Adh transcript.

In an effort to determine the origins of and mechanisms resulting in the sequence length variation observed, we have cloned and characterized each insert. Length variation formed two classes: unique sequence events (DNA involved not present elsewhere in the genome) or repeated sequence events due to the presence of transposable elements. Unique sequence length variation was of two types. Two insertions, present in 30% of the lines, were estimated to be 31 and 34 bp in length. Seven deletions of unique sequence were also observed in 61% of the lines, and ranged in size from 21 to 200 bp.

Seven sizes of inserts, observed in 11 lines, were found to be repeated throughout the genome. A five kb insert, occurring approximately 0.4 kb 5' to the beginning of the adult Adh transcript in one line (RI42), appears to represent a complete copia element while a 4.8 kb insert 3' to Adh in another line appears to be homologous to an F element. Interestingly, all 11 inserts (4 sizes) in the approximately 230 bp EcoRI/SalI fragment 1.5 kb 3' to Adh share sequence homology with a relatively uncharacterized repetitive sequence, termed 2161. Our sequencing shows 2161 to be a member of the F-family of elements by virtue of its structure, in particular, a poly-A end and lack of direct or inverted repeats. The three smaller inserts at this site (340, 400 and 700 bp) have homology to 2161 alone, and represent abbreviated and/or defective copies of a complete element. However, the fourth insert in this EcoRI/SalI fragment (an insert of over 9 kb) shows homology to 2161 at both ends, whereas the central 9 kb represents an apparently complete copy of the element B104. The remaining 3' repetitive insert, "n", appears to represent a 400 bp piece of a B104 element.

One approach to assessing the significance of this enormous amount of sequence variation, most of which occurs in nontranscribed regions, is to ask whether it is associated with altered expression of adjacent genes. A readily assayed index of gene expression at Adh is enzyme activity. While ADH activities are

spread over a five-fold range, two clusters of lines are apparent; termed here high and low activity. Of particular interest is the nearly complete association between the Fast allozyme and high activity, and the Slow with low activity. We were interested in whether the high/low difference was due to the Thr to Lys replacement, which underlies the allozyme difference or perhaps was due to a flanking sequence variant. There are three obvious activity outliers among the lines we have studied; that is allozymes associated with the "wrong" activities. Heat and urea denaturation studies and comparison of activity ratios for different alcohol substrates suggest these lines do not have new amino acid substitutions.

We found strong nonrandom associations among variants in the 12 kilobase region, including the allozyme polymorphism and ADH activity. In addition, activity levels show a pattern generally consistent with the phylogeny constructed for the 49 restriction maps and the distribution of the fast/slow polymorphism. The restriction map for one of the lines with the "wrong" activity for its allozyme (NC16, Fast but low activity) appears to be recombinant in the region just 5' to the Adh allozyme. Another line could be considered either a 5' recombinant or an evolutionary intermediate in the evolution of Fast from Slow. Importantly, recombinants 3' to Adh or possible recombinants in the extreme 5' end of the 12 kb region do not show shifts in ADH activity.

The activity differences for these lines are tightly linked to the allozyme polymorphism and the effect is cis-acting. In addition, the "switch" from high to low activity in NC16 is associated with a switch from high to low level of Adh mRNA and is seen at both the adult and larval stages. In order to test our hypothesis and attempt to refine more closely the precise location of the putative "regulatory" site, we obtained nine D. melanogaster lines from natural populations for which Dr. M. Kreitman had determined the DNA sequence for a 2.5 kilobase region surrounding and including the Adh gene. Our restriction mapping of these lines extended DNA sequence markers to the full 12 kb region and indicated two apparent natural recombinants (or convertants) on the 5' side of the Lys to Thr amino acid replacement. Subsequent determination of ADH activity, however, showed no "switch" in activity relative to the allozyme. Thus, these data (together with our other data) suggest that the nucleotide responsible for the major between-allozyme difference in Adh activity and messenger RNA levels is a third codon substitution contained within the third exon of the ADH protein. The effect on this substitution of standing levels of Adh expression could result from differences in the rate of transcription, the rate of processing the stability of the transcript, or the rate of translation. This is being studied.

No clear pattern of association of gene expression and insertion/deletion variation emerged from an examination of the different types of events as classes. For example, transposable elements are present in the majority of the lines having outlier activities. However, other lines with "normal" activities for their allozyme also have transposable elements, often of similar size and in the same location, suggesting the altered activity does not result from the insertion.

The low activity of one line (RI42) with a 5 kb copia element inserted 400 bp 5' to the start of the adult transcript is tightly linked to the Adh structural gene and cis-dominant. This line also shows an altered developmental pattern of expression, with larval activity disproportionately reduced. This latter observation is notable since the larval transcript initiation site is nearly a kilobase farther from the site of insertion than from the beginning of the adult transcript. In addition to the genetic evidence, however, support for the copia insert as the cause of the altered activity comes from our own mRNA level studies that indicate the activity differences are correlated with mRNA level differences and transformation studies of Posakony and Maniatis that demonstrate that deletions of sequence from this 5' region affects larval activity to a greater extent than adult activity.

Thus, it is clear that alterations in flanking regions can have detectable effects. However, some variation in flanking sequences is not clearly associated with altered levels of adult ADH activity, yet may have significant effects. Only the frequencies of transposable elements showed a clear and significant departure from predictions of selective neutrality. (This frequency observation has held up in our examination of D. melanogaster from populations throughout the world.) Additional theoretical studies indicate the transposable elements (and probably the deletions) are deleterious although with small selection coefficients (several times their rate of insertion). In addition, unlike base substitutions, sequence length variation is not observed to accumulate through evolutionary time. Selection against transposable elements probably relates to the increased mutational load associated with increased copy number in the genome rather than selection against individual sites of insertion although the latter certainly occurs occasionally.

We have documented a hotspot 3' to Adh for the insertion of a new transposable element. This hotspot is correlated with an altered chromatin structure in that region. The DNA sequence of the new element has been determined and it appears to be a new member of the F-family. We have found no open reading frames within the elements or evidence of homology with normal genes for which DNA sequences are available.

The generality of the Adh region results from D. melanogaster are an important question. Thus, we have examined this region in two related species, D. simulans (a close relative) and D. pseudobscura (diverged probably 20 - 30 million years ago). These studies have involved restriction analyses in both species as well as cloning and sequencing 3.5 kilobases in D. pseudobscura including the Adh gene and adjacent 3' sequence. Several notable results emerged. Surprisingly, both simulans and pseudobscura have higher levels of nucleotide polymorphism but lower levels of insertion/deletion variation than D. melanogaster. For simulans at least, these results are consistent with an across-the-board reduction in total copy number of transposable elements. An important question is why does this difference exist? Comparison of our DNA sequence for D. pseudobscura to D. melanogaster has also revealed a new gene starting approximately 130 bp 3' to Adh. The function of this coding sequence remains unknown but its presence provides an explanation for the conservation of this region within and between species that had previously thought to be simply "spacer DNA".

PLANS FOR SUBSEQUENT YEAR: Project terminates with the departure of Dr. Charles F. Aquadro in August, 1985.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The assessment of risk to the human population from exposure to environmental mutagens depends on a solid understanding of population genetics. The potential significance of insertion/deletion variants (particularly transposable elements) in and outside transcriptional units is unclear but the question is approachable. These results contribute to the understanding of domains responsible for proper gene expression and define genetic lesions that can upset that regulation.

PUBLICATIONS

Aquadro, C.F., S.F. Deese, M.M. Bland, C.H. Langley and C.C. Laurie-Ahlberg: Molecular population genetics of the alcohol dehydrogenase region of *Drosophila melanogaster*. Genetics (In Press).

Golding, G.B., C.F. Aquadro and C.H. Langley: Sequence evolution within populations under multiple types of mutation. Submitted to P.N.A.S.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61019-05 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Collaborative Protein Sequencing

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Steven S.-L. Li Research Geneticist LG, NIEHS

Others: Farida S. Sharief Biologist LG, NIEHS

COOPERATING UNITS (if any)

Department of Diagnostic Immunology Research and Biochemistry, Roswell
Park Memorial Institute, Buffalo, New York

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Four acid phosphatases were isolated from normal human lungs, spleens, kidneys and bladders, and their structural and functional properties were compared with that of human malignant prostatic acid phosphatase. These acid phosphatases possess different molecular weights, amino acid compositions, peptide maps, substrate specificity and optimal pH, although they share at least one common antigenic determinant in addition to their own specific sites. The antigenic structure of human prostatic acid phosphatases (PAP) was analyzed by using three partial tryptic peptide fragments. The entire PAP molecule comprises a minimum of four distinguishable, non-overlapping antigenic determinants.

A glycoprotein exhibiting immunological and enzymatic activities of human prostatic acid phosphatase has been purified and amino-terminal sequences of both prostatic acid phosphatase and glycoprotein were found to be different.

The primary structure information of protein is very important in elucidating the fundamental biological function. The collaborative research of protein sequencing provides accurate information that can be used for cloning and identification of eukaryotic genes.

PROJECT DESCRIPTION

PROBLEM: Collaborative protein sequencing of human acid phosphatase isozymes.

OBJECTIVES: The information on the primary structure of proteins is very important in elucidating the structure-function relationship of proteins. The collaborative research of protein sequencing provides fast and accurate information that can be used for cloning and identification of eukaryotic genes.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: The prostate cancer is second-largest killer among men, after lung cancer. Since elevated activity of serum acid phosphatase is a combination of many isozymes from various tissues and blood cellular components, several immunoassays based on the immunologic specificity of prostatic acid phosphatase have been developed as a diagnostic marker for the detection of PAP levels in serum from patients with prostate cancer. In order to determine the complete primary and antigenic structures as well as to study the regulation of their expression, the partial amino acid sequences of human acid phosphatases will be determined for the synthesis of oligonucleotides used as probes to clone and identify their genes.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: In collaboration with Dr. T.-M. Chu of Roswell Park Memorial Institute, four acid phosphatases were isolated from normal human lungs, spleens, kidneys and bladders, and their structural and functional properties were compared with that of human malignant prostatic acid phosphatase. These acid phosphatases possess different molecular weights, amino acid compositions, peptide maps, substrate specificity and optimal pH, although they share at least one common antigenic determinant in addition to their own specific sites. The antigenic structure of human prostatic acid phosphatases (PAP) were analyzed by using three partial tryptic peptide fragments. The entire PAP molecule comprises a minimum of four distinguishable, non-overlapping antigenic determinants.

Two prostatic acid phosphatase isozymes (PAP-I & PAP-II) and a glycoprotein (GP) which is immunochemically and biologically related to PAP, have been purified and partially characterized from human seminal plasma. PAP-I possess two subunits of 50,000 each in comparison with subunits of 55,000 for PAP-II. The GP has an apparent molecular weight of 45,000. The purified GP was shown to exhibit a weak, but significant, acid phosphatase activity. Amino acid compositions, peptide maps and carbohydrate contents of PAP-I, PAP-II and GP have been obtained. Amino-terminal sequences of PAP-I and GP have also been determined. These chemical data as well as immunological results demonstrate that PAP-I and PAP-II are different human PAP isozymes and that the GP represents a distinct glycoprotein which shares some common enzymatic and antigenic characteristics with PAP. In Dr. T.M. Chu's laboratory monoclonal antibodies prepared against prostatic acid phosphatase have been obtained and immunotoxins using ricin and abrin are being developed to cure the human prostate cancer.

FUTURE RESEARCH PLANS: Acid phosphatase isozymes from human tissues such as lungs, spleen, kidneys and bladder are purified and compared with those of prostatic acid phosphatase isozymes. Amino-terminal sequences for all acid phosphatase isozymes purified will be determined, and some of their peptides will be isolated and sequenced.

PUBLICATIONS

Lin, M.F., Lee, C.L., Li, S.S.-L., and Chu, T.M.: Purification and characterization of a new human prostatic acid phosphatase isozyme. Biochemistry 22: 1055-1062, 1983.

Lin, M.F., Lee, C.L., Sharief, F.S., Li, S.S.-L., and Chu, T.M.: Glycoprotein exhibiting immunological and enzymatic activities of human prostatic acid phosphatase. Cancer Research 43: 3841-3846, 1983.

Lee, C.L., Li, S.S.-L., and Chu, T.M.: Immunologically reactive tryptic fragments of human prostatic acid phosphatase. Biochemical Journal 223: 871-877, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61021-04 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic and Molecular Analysis of the cut Locus of D. melanogaster

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Joseph W. Jack Senior Staff Fellow LG, NIEHS

Other: Willie Gibson Research Chemist LG, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

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Eukaryotic Gene Structure Section

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NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are interested in knowing how cells of a single organism can differentiate to form specific tissue types. We have chosen to address one aspect of the question by learning how one gene, the cut locus of Drosophila melanogaster, is expressed differently in different tissues of the fly.

We have now cloned DNA sequences representing the entire gene, which encompasses 200 kb or more of DNA. A large number of mutants have been analyzed. We find that the deletion of sequences in the leftmost part of the gene cause phenotypic effects primarily in the legs, while deletion of or insertions into sequences slightly to the right cause effects primarily in the wings. Mutations in a 70 kb to the right affect the wings, head, and thorax and cause lethality. A fourth group of mutations lacks cut locus function in all tissues. These mutations map at the rightmost end of the gene.

The availability of tissue specific mutants of a gene afford the opportunity to experiment to find out how the gene normally operates in tissue specific ways. We are currently studying the transcriptional activity of the cut locus to find out how the tissue specificity of the mutant phenotypes relates to the transcriptional activity of the gene.

We now know that many of the cut mutants are insertions of retrovirus-like sequences into the cut locus DNA, and we are interested in understanding the affect of these sequences on gene activity. Some of these mutations are suppressible and will be useful in determining how a mutation caused by a retrovirus-like sequence can be suppressed.

PROJECT DESCRIPTION

MAJOR FINDINGS AND PROPOSED COURSE: In the past year we have completed the cloning of all or most of the DNA comprising the cut locus. The gene is very large, containing 200 kb or more of DNA. Despite its extraordinary size, cut sequences tested have been restricted to a single band on a polytene chromosome. This observation relates to the question of how genes are organized on chromosomes. It has long been noted that complementation groups in Drosophila are frequently associated in a one-to-one fashion with polytene chromosome bands. However, the question of whether these bands represent functional units remains an open question. In this context it is remarkable that although the average band contains about 20-30 kb of DNA, the entire 200 kb of cut locus is limited to a single band.

Three types of cut locus mutants have been recognized for some time. Our genetic analysis of a group of lethal mutations recovered from a strain of mutable flies has identified a fourth type of cut mutation. The cloned sequences have been used to analyze more than 40 mutants. Based on the location of transposable element mutations and the extent of deficiencies and an inversion, four cut locus regions can be defined. Mutations in the most distal 40 kb cause leg defects. More proximal, a region of 20 kb contains mutations that alter the wings and head. Within this region a higher level of organization can be detected. All of the leaky mutants in the region map to a small segment, possibly only 0.5 kb, at the centromere-distal end. Another 50 kb proximal to the wing mutants is a small region of lethal mutations that complement the leg mutations but fail to complement other cut alleles. Finally, the most proximal part of the gene contains lethal mutations that fail to complement all cut locus mutations. This region probably extends to a point at least 60 kb proximal to the rest of the gene.

We have studied the nature of cut locus mutations generated in a mutable strain of flies. These mutations are of the wing specific and lethal varieties. We find that most of these mutations are caused by the insertion of the retrovirus-like transposable element gypsy into cut locus DNA. Thus, the gypsy element apparently transposes exceedingly frequently in the mutable strain, causing mutations at least in the cut locus. Many mutations associated with the insertion of gypsy elements at cut and other loci are known to be suppressed by the mutation of another gene, suppressor of Hairy wing. We are interested in knowing what effect transposable elements exert on genes to prevent their normal functioning. The gypsy element insertions are particularly useful because of the opportunity to suppress the effect of the mutation. By characterizing the location of gypsy elements with respect to cut locus transcripts, their effect on transcription, and their ability to be suppressed, we expect to learn more about the effect of the elements on gene activity and the suppression of that effect. We have now tested a representative sample of gypsy mediated cut alleles for their ability to be suppressed by su(Hw). We find that all are at least partially suppressed.

The analysis of cut locus transcription will explain how these transposable element insertions interrupt expression of the gene and how the effect of gypsy insertion can be relieved by the suppressor mutation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Our analysis of the cut locus is aimed at understanding how genes are regulated during the development of a higher organism and how the process of regulation can be altered by various types of mutations. The analysis of the changes in cut locus activity caused by retrovirus-like sequences will be important for understanding how such sequences affect genetic activity. This understanding will be important since retroviruses are known to alter the behavior of cellular genes, in some cases giving the altered cells the potential to form tumors.

PUBLICATIONS

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61022-04 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Population Genetics of Transposable Elements

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. H. Langley Research Geneticist LG, NIEHS

Others: Elizabeth A. Montgomery Bio. Lab. Tech. LG, NIEHS

COOPERATING UNITS (if any)

Drs. N. Kaplan T. Darden, and R. Hudson, Biometry and Risk Assessment Program
Dr. Brian Charlesworth, Department of Biology, University of Chicago
Dr. Wolfgang Stephan, Department of Genetics, University of Edinburgh

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of natural selection against the deleterious effects of individual transposable element insertions was investigated by comparing the numbers of transposable elements on the X chromosomes versus autosomes of *Drosophila*. There is little evidence for selection against copia-like elements on the X chromosomes. This suggests other possible mechanisms (e.g. chromosome rearrangement) are likely to be important in removing transposable elements from natural populations.

The role of defective transposable elements in the evolution and ultimate extinction of transposable element families was investigated and related to the species distributions of various *Drosophila* transposable elements. A second study investigated the theory of the evolution of copy number regulation of a transposable element in an outbreeding population. The prediction of this study is that dominant effects, such as chromosome rearrangements, are likely to be the events that ultimately make the evolution of copy number regulation likely. Finally, the theory of the evolution of reduced recombination in centromeric and telomeric regions was developed and the associated accumulation of tandemly repeated DNA was investigated.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: The existence of transposable elements (TEs) has been documented in a number of both prokaryotic and eukaryotic organisms. There is a growing body of evidence linking transposable elements with spontaneous mutations in *Drosophila* and other organisms. Little is known, however, of the occurrence and effects of these elements in natural populations. What are the roles of the various forces (e.g. mutation, random genetic drift, transposition, recombination, infection, and natural selection) in the evolution of transposable elements in outbreeding populations?

OBJECTIVES: We are seeking to accumulate data on the natural history of this class of DNA parasites in *Drosophila melanogaster* and in related species. While some transposable element insertions cause visible mutations of major effect, the effects on the host of the vast majority of TE's is not known. A primary objective during the last year has been to examine experimentally and theoretically the forces that contain and ultimately lead to the extinction of transposable elements. If natural selection removes individual insertions because of their deleterious effect on adjacent genes then X chromosomes (as compared to autosomes) would be expected to have fewer elements since recessive effects would be exposed in the hemizygous males. If recombination is important in allowing selection to operate on the individual insertion rather than the whole chromosome, then balanced inversions would be expected to accumulate elements relative to chromosomes that can recombine. Other objectives were the theoretical studies of the evolution of copy number regulation of transposable elements, the role of defective elements in the evolution and ultimate extinction of transposable elements, and finally the evolution of reduced recombination and associated accumulation of tandemly repeated DNA in centromeric and telomeric regions of the chromosome.

EXPERIMENTAL DESIGN AND SCIENTIFIC JUSTIFICATION: The family of transposable elements in *Drosophila* known as copia-like elements bears a strong resemblance to vertebrate retrovirus sequences. This and other families of transposable elements in *Drosophila*, such as the P element, are responsible for spontaneous mutations. The identification, cloning and characterization of these elements from *D. melanogaster* affords us the opportunity, using molecular and cytological techniques, to learn about the population biology of the elements and their effects on the organisms they inhabit. Both experimental and theoretical tools have been used to approach these problems.

The question of the effect on the host of TE's in the absence of obvious mutations can also be tested. The hypothesis that the elements may individually have mildly deleterious effects was tested. *Drosophila melanogaster* chromosomes, genetically isolated from a natural population, were probed with cloned TE's *in situ*. Comparisons were made for each element between the number present on the X chromosome versus the number on the autosomes. The numbers of transposable elements in balanced inversion chromosomes that have been maintained in the laboratory for fifty years was compared to those in chromosomes that were not balanced. This was done by *in situ* hybridization of many transposable element sequences to *Drosophila* chromosomes.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: The results of the survey of the numbers of transposable elements (297, 412 and B104) on the X, 2nd and 3rd chromosomes (20 each) from a natural population showed that X chromosomes have proportional numbers of transposable elements when compared to autosomes. This indicates that natural selection against individual insertions is not the major force removing these elements from the population. Preliminary surveys of numbers of elements in balanced inversions indicated increased numbers relative to comparable unbalanced controls.

The theoretical analysis of the evolutionary consequences of defective elements to the stability of a transposable element family showed that they can have a profound effect on the likelihood that the family ever goes extinct. A theory of the evolution of copy number regulation was developed that predicts that dominant effects associated with transposition are likely to be those that lead to the evolution of copy number regulation. Finally, the consequences of the emerging molecular models of centromeric and telomeric function and organization for the evolution of restricted recombination in these regions was explored theoretically. The analysis predicts the accumulation of tandemly repeated sequences in these regions.

PLANS FOR SUBSEQUENT YEARS: The survey of transposable elements on balanced inversions will be continued. But the major effort will go into the investigation both experimentally and theoretically of the hypothesis that the primary mechanism that controls copy number is the recombination between elements at nonhomologous sites in the genome leading to aneuploid germ cells. These events are rare. A selective scheme has been devised to collect the products of such events from wild *Drosophila* so that the breakpoints can be characterized. The theoretical consequences of this hypothesis will be investigated. Prediction from this model will be examined to see if it can be distinguished from alternatives.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Mounting evidence suggests that many "spontaneous" mutations are due to insertion of transposable elements. The insertion of transposable-element-like retrovirus sequences are implicated in oncogenesis. The understanding of the population biology of these chromosomal parasites will be essential in evaluating population risk to both mutagenesis and cancer.

PUBLICATIONS

Kaplan, N., Darden, T. and Langley, C.H. Evolution and extinction of transposable elements in Mendelian populations. Genetics 109:459-480, 1985.

Charlesworth, B., Langley, C.H. and Stephen, W. The evolution of restricted recombination and the accumulation of repeated DNA sequences. (submitted to Genetics), 1985.

Charlesworth, B. and Langley, C.H. The evolution of self-regulated transposition of transposable elements. (submitted to Genetics), 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61023-03 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of Drosophila Germ Cell Determination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert E. Boswell Staff Fellow LG, NIEHS

Others: Marcia Meltzer Microbiologist LG, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

1.75

PROFESSIONAL:

1.75

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is a fundamental concept in developmental biology that the fate of embryonic cells is regulated by morphogenetic determinants localized in the ooplasm. In *Drosophila*, heterotopic transplantation experiments have conclusively demonstrated that cytoplasmic factors localized to the posterior pole plasm of the oocyte and embryo are requisite for the formation of pole cells, the primordial germ cells. However, the molecular nature of these cytoplasmic factors, the mechanism of localization within the ooplasm, and their mode of action in development are unknown. The genetic and developmental analysis of maternal effect mutants that affect pole cell formation in *Drosophila melanogaster* are intended to allow one to elucidate the mechanism of determination and how the determined state is maintained throughout development.

A detailed genetic and developmental analysis of one such grandchildless mutant, tudor, (tud) has been undertaken. The properties of mutations of the recessive maternal effect gene tud indicate that the gene product of the tudor locus is required for the proper determination of germ cells in *Drosophila melanogaster*. Specifically, the germ plasm of six different alleles of tud has been analyzed at the ultrastructural level, and it is found that different alleles contain different amounts of assembled polar granule material (a cytoplasmic organelle classically thought to be the germ cell determinants). The ability or inability to form germ cells correlates directly to the amount of assembled polar granule material observed in the germ plasm. For example, one allele produces polar granules approximately 1/3 the size of wild type polar granules and this allele produces fertile progeny. On the otherhand, alleles that produces no apparent assembled polar granule material in the germ plasm produce no fertile progeny. Therefore, mutations at the tudor locus disrupt the normal assembly of the germ plasm resulting in the failure to localize the germ plasm determinants to the posterior pole.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: It is a fundamental concept in developmental biology that the fate of embryonic cells is regulated by morphogenetic determinants localized in the ooplasm. In Drosophila, heterotopic transplantation experiments have conclusively demonstrated that cytoplasmic factors localized to the posterior pole plasm of the oocyte and embryo are requisite for the formation of pole cells, the primordial germ cells. However, the molecular nature of these cytoplasmic factors, the mechanism of localization within the ooplasm, and their mode of action in development are unknown.

OBJECTIVE: An understanding of the molecular mechanisms involved with germ cell determination would be a significant contribution to our understanding of developmental processes. The aim of this research then is to determine (1) the molecular nature of the determinants required for specifying the fate of a particular cell type (the primordial germ cells), (2) how these molecules specify and maintain the particular developmental commitment undertaken by these cells and (3) the means by which these informational molecules become localized to a discrete cytoplasmic site within the egg.

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: The analysis of germ cell determination in Drosophila will require a combined genetic, developmental and molecular characterization of the process. The strategy is to isolate and characterize selected grandchildless loci. These loci will be characterized to determine the nature of the developmental lesion induced by disrupting the normal gene function. The available technologies (e.g. recombinant DNA, sequencing and antibodies) will be used with the intent of isolating the gene products and establishing their roles in germ cell determination.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: A new maternal effect grandchildless (gs) mutant, tudor, has been analyzed. New alleles of tudor were recovered by X-ray mutagenesis. The posterior pole plasm of oocytes and preblastoderm embryos was analyzed by transmission electron microscopy (TEM).

Homozygous tudor females produce embryos that lack pole cells and the adults of both sexes are sterile. The germ plasm produced by six different alleles of tudor has been analyzed ultrastructurally by TEM. Oocytes or embryos from each genotype contain different amounts of assembled polar granule material, such that they form a continuum from those that contain no apparent assembled polar granule material to types that contain polar granules approximately 1/3 the size of polar granules observed in the wild type oocyte or embryo. The ability or inability of any given allele to produce pole cells (the primordial germ cells) correlated directly with the amount of assembled polar granule material in the germ plasm. For example, the allele (tud¹) produces oocytes and embryos with polar granules approximately 1/3 the size of wild type polar granules and is fertile as a homozygote. However, all alleles containing polar granules less than 1/3 the size of wild type polar granules or no apparent polar granule

material are sterile as homozygotes. This indicates then, that mutations at the tudor locus disrupt the normal assembly of the germ plasm in a quantitative fashion, different alleles contain different amounts of polar granule material. It is postulated that the failure to properly assemble the germ plasm disrupts the localization of the germ cell determinants thereby resulting in a failure to form germ cells.

Like most maternal effect mutants studied to date, tudor is pleiotropic, and approximately 40% of the embryos from the homozygous gs females die during embryogenesis and exhibit segmentation pattern abnormalities. The remaining 60% of the embryos are phenotypically normal, but they are sterile due to a failure in germ cell formation. The severity of the segmentation pattern abnormalities and the sterility due to a failure in pole cell formation correlate with the amount of assembled polar granule material in the germ plasm. For example, tudor⁺ is not grandchildless as a homozygote, but is grandchildless when heterozygous with tudor alleles containing reduced amounts of polar granule material in the germ plasm. This leaky allele expresses few, if any, of the segmentation pattern abnormalities observed in embryos derived from homozygous females of tudor alleles containing no or reduced amounts of polar granule material assembled in the germ plasm.

These properties of mutations at the tudor locus indicate that the gene product of the tudor locus is required for the determination of germ cells and for the normal pattern of segmentation in Drosophila melanogaster. In particular, the tud⁺ gene product appears to be a component necessary for the proper assembly of the germ plasm.

Various X-ray induced alleles of tudor are chromosomal rearrangements. Deletion mapping taken together with the cytological mapping of the chromosomal rearrangements place the tudor locus in the right arm of the second chromosome within the polytene chromosome interval 57C8-9. Recently, a unique sequence segment of Drosophila melanogaster DNA cloned into the λ cloning vector Charon 4 has been found to be homologous to the polytene chromosome interval 57C. Using standard molecular and cytogenetic techniques we have determined the position of this cloned segment of Drosophila melanogaster DNA relative to the tudor locus. We have walked approximately 160 kbp from the site of entry towards the tudor locus.

PLANS FOR SUBSEQUENT YEARS: New alleles of tudor and loci flanking the tudor locus will be obtained using a variety of mutagens (X-ray, diepoxybutane, N-ethyl-N-nitroso-urea, ethylmethane sulfonate). The pole plasm of oocytes and preblastoderm embryos will be analyzed by transmission electron microscopy (TEM). Standard molecular techniques will be used to obtain recombinant DNA clones containing the tudor structural gene.

The genetic analysis of tudor and other grandchildless loci will allow one to study the cytological consequences of genetic lesions leading to grandchildless phenotype. These studies will require TEM of the pole plasm of stage 13-14 oocytes as well as preblastoderm embryos. Pole cells of blastoderm embryos will

also be analyzed cytologically. Scanning electron microscopy (SEM) will be used to analyze any morphological defects in developing embryos that may be associated with the abnormal segmentation. Segmentation defects can also be studied using acetylcholinesterase whole mounts and plastic sections of whole embryos. The segment defects will also be studied using Hoyer's mounts to identify the cuticle pattern defects.

The chromosomal walk undertaken to isolate the tudor structural gene will be continued. Chromosome rearrangements associated with the tudor locus will serve as molecular landmarks to define a region of the genome that must be intact for the proper expression of the tudor locus. Furthermore, we have several small deficiencies (approximately 5 chromosomal bands) that define the left and right most boundaries of the tudor locus.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A major interest within the institute is to understand the mechanisms involved in cellular determination and differentiation. Elucidation of the molecular events in determination is considered crucial to understanding medical problems such as cancer and aging.

PRIORITY NEEDS: The developmental analysis of tudor mutations will require ready access to a transmission and scanning electron microscope. A completion of the chromosomal walk to clone the tudor locus would most readily be accomplished with continued technical assistance.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61024-03 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic and Molecular Analysis of Suppressor-of-Sable Function in *Drosophila*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. A. Voelker Research Geneticist LG, NIEHS

Others: G. B. Wisely Bio. Lab. Tech. LG, NIEHS
J. F. Sterling Bio. Lab. Tech. LG, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

4.6

PROFESSIONAL:

2.8

OTHER:

1.8

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent findings in *Drosophila* have shown that 1) a significant proportion of spontaneous mutations are caused by insertions of mobile genetic elements, and 2) certain genetic suppressor systems are mediated through insertions of specific mobile elements. We are investigating the molecular mechanism of one such suppressor system: recessive mutations at the suppressor-of-sable [su(s)] locus suppress recessive mutations at the vermilion (v) locus that are caused by insertions of the mobile elements 412 and B104 (Project Number Z01 ES 61029-02 LG).

DNA sequences of su(s) have been cloned and are being characterized. The insertions of foreign DNA that are associated with 14 su(s) mutant alleles have been localized to a region of DNA that encodes the 5' end of the message. The DNA sequences that give rise to the ~5 kb poly A⁺ su(s) message consist of at least 5 exons interspersed over 8 kb of genomic DNA. Coding sequences from the two largest exons will be ligated into an expression vector to produce a fusion protein, against which antibodies can be produced. Antibodies against the su(s) portion of the fusion protein will be recovered and used as probes to identify the location and function of the su(s) protein product within the organism. The interaction of the su(s) protein with the v locus will be studied to determine how suppression is effected. By gaining an understanding of this phenomenon, we will learn if this type of suppression mechanism is an adaptive feature of *Drosophila* to deal with mutations caused by mobile element insertions.

PROJECT DESCRIPTION

PROBLEM: Recent findings have shown that a significant proportion of spontaneous mutations in *Drosophila* are caused by insertions of mobile genetic elements, and that certain genetic suppressor systems are mediated through insertions of specific mobile elements. We are investigating the molecular mechanism of one such suppressor system. Recessive mutations at the suppressor-of-sable [su(s)] locus suppress recessive mutations at the vermilion (v) locus that are caused by insertions of the mobile elements 412 and B104.

OBJECTIVES: A primary goal of the work is to determine the protein product of su(s), a reduction or an absence of which effects suppression of several v alleles. Coding sequences from su(s) will be ligated into an expression vector to produce a fusion protein, against which antibodies can be produced. Antibodies against the su(s) portion of the fusion protein will be recovered and used as probes to identify the location and function of the su(s) protein product within the organism. The interaction of the su(s) protein with the v locus will be studied to determine how suppression is effected. By gaining an understanding of this phenomenon, we will learn if this type of suppression mechanism is an adaptive feature of *Drosophila* to deal with mutations caused by mobile element insertions.

METHODS EMPLOYED:

- 1) Duplication/deficiency mapping and genetic complementation tests will be used to analyze the mutants recovered from a lethal saturation mapping of the su(s) region.
- 2) The P-element transposon-tagging technique has been used to clone su(s).
- 3) Conventional endonuclease restriction mapping is being used to characterize the structure of wild type and mutant DNA sequences.
- 4) Northern blot analyses of polyA⁺ RNA's are being used to map the extents of coding sequences.
- 5) M13 single-stranded probes are being used to identify the direction of transcription.
- 6) S1 nuclease-type mapping methods are being used to locate the intron and exon components of the coding sequences.
- 7) The structure and function of the su(s) protein product are completely unknown; therefore, a fusion protein expression vector system will be used to express and recover a hybrid protein from which to make and recover antibodies which will be used to recover and localize the entire protein.
- 8) The DNA sequences of wild type and su(s) mutant alleles will be sequenced to determine the locations of the mobile element insertions with respect to intron and exon sequences.

MAJOR FINDINGS AND PROPOSED COURSE:

- 1) The P-element transposon tagging technique has been used to clone DNA sequences of su(s).
- 2) Using wild type DNA sequences of su(s) as probes, the restriction maps of 23 su(s) mutant alleles have been analyzed.
 - a) All of seven spontaneous mutations contain insertions of non-su(s) DNA sequences; many of these inserted DNA's are mobile genetic elements.
 - b) None of nine X-ray or EMS-induced mutations contain altered restriction patterns;
 - c) All of five hybrid dysgenesis-induced mutations contain insertions of non-su(s) DNA sequences.
 - d) Two of two DNA transformation-induced mutations contain insertions of non-su(s) DNA sequences.
- 3) A ~5 kb polyA⁺ message is homologous with sequences spanning approximately 8 kb of DNA from the su(s) region; this suggests the presence of sizeable introns.
- 4) The direction of transcription of su(s) is distal to proximal (telomeric → centromeric);
- 5) S1 protection mapping has identified at least 5 exons of the following sizes: 2.5 kb, 1.35 kb, 0.4 kb, 0.4 kb and 0.2 kb; other smaller exons may yet be detected. The three smallest exons comprise the 5' end of the message and it is in DNA encoding this portion of the message that the above mentioned DNA insertions occur.
- 6) An exon from the su(s) region will be ligated into an expression vector to produce a su(s)-β galactosidase fusion protein. The fusion protein can be precipitated with commercially available anti-β-galactosidase antibodies. The precipitate can be injected into rabbits to produce antibodies which can be fractionated to recover those specific for the su(s) component of the fusion protein. These su(s)-specific antibodies can be used as probes for the structural location and function of the su(s) protein product.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is becoming apparent that mobile genetic elements are responsible for many so-called spontaneous mutations. Since most mutations are harmful, such mutations contribute to the genetic loads of populations and in humans these mutations become causes of genetic diseases. Thus, an understanding of how these mobile genetic elements cause mutations and how an organism may evolve systems to suppress these mutations offers the prospect of minimizing the effects of such mutations or perhaps eventually elimination them.

PUBLICATIONS

Voelker, R.A., Chang, D.-Y., Huang, S.-M., and Wisely, G.B.: Cloning and molecular characterization of the suppressor-of-sable [su(s)] gene from *Drosophila*. Abstract. Genetics 107: 111-112, 1984.

Voelker, R.A., and Wisely, G.B. Suppressor of sable [su(s)] transcript mapping in *Drosophila melanogaster*. Abstract. Genetics, (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61025-02 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

DNA Sequence Variation in the Dopa Decarboxylase Region of Drosophila

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Charles F. Aquadro Staff Fellow LG, NIEHS

Others: Charles H. Langley Research Geneticist LG, NIEHS

COOPERATING UNITS (if any)

Dr. C. Laurie-Ahlberg, Associate Professor of Genetics
North Carolina State University, Raleigh, North Carolina

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Study continued under Project Number Z01 ES 61036-01 LG entitled "Naturally occurring DNA Sequence Variation in Drosophila melanogaster".

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61027-02 LG			
PERIOD COVERED October 1, 1984 to September 30, 1985					
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Transmission of Cryptic Mutations in Destabilized X Chromosomes of <i>Drosophila</i>					
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> PI: B. H. Judd J. W. Jack </td> <td style="width: 33%; vertical-align: top;"> Research Geneticist Senior Staff Fellow </td> <td style="width: 33%; vertical-align: top;"> LG, NIEHS LG, NIEHS </td> </tr> </table>			PI: B. H. Judd J. W. Jack	Research Geneticist Senior Staff Fellow	LG, NIEHS LG, NIEHS
PI: B. H. Judd J. W. Jack	Research Geneticist Senior Staff Fellow	LG, NIEHS LG, NIEHS			
COOPERATING UNITS (if any) Dr. Johng K. Lim, Professor of Biology University of Wisconsin, Eau Claire					
LAB/BRANCH Laboratory of Genetics					
SECTION Eukaryotic Gene Structure Section					
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709					
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:			
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews					
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Study continued under Project Number Z01 ES 65037-01 LG entitled "Transposon - mediated chromosome instabilities in <i>Drosophila</i> ".					

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61029-03 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cloning and Characterization of the vermilion Locus of Drosophila

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Lillie L. Searles Senior Staff Fellow LG, NIEHS

Others: Robert A. Voelker Research Geneticist LG, NIEHS
Mary L. Tate Bio. Lab. Tech. LG, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objectives of this project are to clone the vermilion locus of Drosophila, to study the structure and expression of the gene, and to investigate the nature of vermilion mutations that can be suppressed by mutating the suppressor of sable locus.

Vermilion encodes the enzyme tryptophan oxygenase which catalyzes the first step in the synthesis of the brown eye pigment. DNA from this locus was cloned from a mutant containing a P element insertion at vermilion by "transposon tagging." The cloned DNA hybridizes to the polytene chromosome band that contains vermilion. Furthermore, DNAs from a number of vermilion mutants show detectable aberrations in the region homologous to the cloned DNA.

Several spontaneous mutations at vermilion, sable, purple, and speck are suppressible by mutations at suppressor of sable. Using cloned vermilion DNA probes, we have determined that these suppressible vermilion alleles are DNA insertion mutations, and DNAs from several of these mutants have been cloned. The mutants v^- , v^k and v^+ are insertions of the copia-like element known as 412. The weakly suppressible mutant v^- contains an insertion of the element known as roo or B104.

The vermilion transcript, 1.4 kb long, originates from the same DNA region where the mutations occur. Transposable element insertion mutations disrupt the production of this transcript. Work is continuing to determine how this disruption occurs and how suppressor of sable restores function to the locus.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: Molecular studies of the vermillion locus of *Drosophila* have been undertaken in order to address questions relating to the control of gene expression in higher organisms. The vermillion gene, whose product is required for the first step in brown eye pigment synthesis, is located on the X-chromosome at cytological band 10A1,2. One class of vermillion mutants is suppressible by the suppressor of sable locus. We have recently shown that these suppressible vermillion mutations are insertions of a retroviral-like transposable element. In *Drosophila*, there are several different suppressors of the same type; each relieves the effects of mutations due to a specific transposable element. Suppression of retroviral-like transposable element insertion mutations has also been observed in yeast and the mouse. Yet, the mechanisms of suppression are not understood in any of these systems. Since vermillion is a relatively simple, well-defined system, these studies will hopefully be informative about how insertions of retroviral-like transposable elements at or near a gene affect gene expression. Furthermore, it is possible that studies of these suppressors and suppressible mutations might be informative about gene regulation in general, as studies of suppressors in bacterial systems have been.

In addition to studying suppression of transposable element insertion mutations at vermillion, we will investigate the molecular basis for developmentally specific vermillion gene expression. Enzyme activity measurements indicate that the vermillion gene product, tryptophan oxygenase, begins to accumulate late during the larval stage, culminating in a peak of activity during the pupal stage. There is a second peak of activity in the adult stage. Enzyme localization studies have shown that the larval activity is restricted to the fat body and that a large proportion of the adult activity is localized in the head. We intend to determine how the expression of the vermillion gene is specified to occur at a given time and in specific tissues.

OBJECTIVES: The goals of this project are to define the structure of the vermillion gene and to determine how it is expressed normally throughout development. The effects of mutations on gene structure and activity as well as the effects of suppressor of sable on gene activity of mutants will be investigated.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: We have cloned the vermillion gene and analyzed DNA from mutants to determine the types of changes that have occurred and to localize the mutation sites. We are also using cloned DNA to characterize vermillion gene expression at the level of transcription in wild type, mutant and suppressed vermillion flies. This correlation of changes in DNA structure with changes in gene activity will contribute to the understanding of how retroviral-like transposable elements affect gene expression.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE TO BIOMEDICAL RESEARCH: We cloned vermilion (v) initially using a mutant (v^Hd) tagged with the transposon P factor. Subsequently, we have cloned DNA from wild-type flies spanning approximately 30 kilobase pairs (kb) in the vermilion region, and we have cloned DNA from several vermilion mutants. From the analyses of DNA from various mutants we have determined that the physical map of DNA in the region cloned is consistent with predictions based on fine structure genetic recombination studies. The size of the gene, defined by mutations, is small. All of the mutations which are detectable as changes in DNA restriction patterns are clustered within approximately a 2 kb region. The vermilion transcript, 1.4 kb in length, is synthesized from this same 2 kb region where the mutations occur. Thus, it seems that, as expected, the vermilion locus is a relatively simple genetic system which should be well suited for studies of suppression and gene regulation.

Three spontaneous vermilion alleles (v¹, v², and v^k) as well as certain spontaneous alleles at the functionally unrelated loci sable, purple and speck, are suppressible by suppressor of sable [su(s)]. A fourth vermilion mutant v^{36f}, is reported to be slightly affected by su(s), although the effect is too slight to alter the phenotype. The interaction between vermilion and suppressor of sable has been investigated in previous years by analysis of phenotypes and measurements of tryptophan oxygenase, the vermilion gene product, activity. These studies have demonstrated that when su(s) is expressed normally and a mutation (v¹, v², v^k) is present at the v locus, tryptophan oxygenase activity is absent and a mutant phenotype is observed. On the other hand, recessive mutations at su(s) partially restore tryptophan oxygenase activity and completely suppress the mutant phenotype although the mutation at vermilion persists; thus, suppression is the consequence of a loss of function at the su(s) locus. While these studies provided a good description of the interaction between these two genes, the mechanism of suppression remained a mystery.

Our analysis of suppressible vermilion mutations at the molecular level is already contributing to the knowledge of suppression. The three suppressible mutations v¹, v², v^k are insertions of the retroviral-like element known as 412. This 7.5 kb element, previously characterized by other labs, consists of a central core sequence bounded by a 480 base pair direct repeat at each end. There are approximately 40 copies of 412 distributed throughout the genome, the sequences being closely conserved at each site. The v^{36f} mutation is an insertion of the 9 kb roo element, which is also similar in structure to retroviruses. We have preliminary evidence, based on in situ hybridization to sable, purple, and speck mutant chromosomes, that the suppressible purple and speck mutations may also be 412 insertions. Thus, we have demonstrated that suppressor of sable belongs to the class of Drosophila suppressors that suppress specific transposable element insertion mutations. Included in this group are suppressor of Hairy wing and suppressor of forked, which suppress gypsy element insertion mutations and suppressor of white-apricot which suppresses a copia element insertion.

To further characterize vermilion and suppression we are currently analyzing transcription in the vermilion region. Two transcripts have been detected within the 30 kb of cloned DNA. A 2 kb transcript is the product of an unidentified gene located immediately to the right of vermilion. This transcript is present at the same level irrespective of the vermilion genotype of RNA. The 1.4 kb vermilion mRNA is transcribed from the same region where v mutations are located. The X-ray induced mutant v^{48a}, which is a 200 base pair deletion within the gene, produces a v transcript at a normal level that is slightly smaller in size than wild type. A v transcript is not detectable in polyadenylated RNA isolated from the mutants v₁, v₂, v^k and v_{36f}. Thus, these transposable element insertions disrupt transcription of the vermilion gene, though the manner by which this disruption occurs has not yet been determined. Preliminary results indicates that a small amount of apparently normal-sized transcript is made from the vermilion locus in suppressed vermilion flies.

We are also currently engaged in a more detailed study of the vermilion transcript. Preliminary nuclease mapping studies indicate that this small gene consists of 6 or more intervening sequences (introns). We want to determine the exact positions of intron and exons, the 5' and 3' ends of the gene and to determine where the transposable element insertions lie relative to transcribed sequences. Studies with other suppressors indicate that suppressible transposable element insertion mutations occur in 5' non-coding regions and introns. We will determine if this is also the case with the suppressible 412 insertion mutations at vermilion.

In mammals, retroviral DNA insertions are implicated in alteration of the expression of nearby genes in some cases leading to cancer. Perhaps the information obtained from studies of the effects of similar DNA insertions on gene expression in *Drosophila* might provide insight into similar processes in higher organisms.

PLANS FOR SUBSEQUENT YEAR: We will continue to investigate transcription of the vermilion locus. The exact position of introns and exons will be mapped; wild-type and mutant genes will be sequenced. From these studies we should obtain a clear picture of what the gene looks like and where suppressible mutations lie relative to coding sequences. We will also characterize transcription of suppressed vermilion flies in detail.

One common feature of retroviral-like elements is that they are abundantly transcribed. A model proposed from studies of other suppressor systems suggests that transcription originating or terminating within the retroviral-like elements interferes with gene activity at affected loci and that suppressors alter transcription of the element. We will test this hypothesis at vermilion. Furthermore, we will determine what general effect if any su(s) has on transcription of 412 elements. In addition, we will begin to analyze the developmental profile of vermilion gene activity.

PUBLICATIONS

Searles, L.L. and Voelker, R.A.: Molecular Characterization of the *Drosophila* vermilion Locus and Its Suppressible alleles. Soon to be submitted to Cell.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61030-02 LG
PERIOD COVERED October 1, 1983 to September 30, 1984		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular analysis of the <u>Om</u> mutator in <u>Drosophila ananassae</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: C. H. Langley Research Geneticist LG, NIEHS Others: Elizabeth A. Montgomery Bio. Lab Tech. LG, NIEHS Antony Shrimpton Visiting Fellow LG, NIEHS		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Genetics		
SECTION Eukaryotic Gene Structure Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.7	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Hinton (1984) described an unusual mutator in <u>D. ananassae</u> which he speculated was a transposable element, <u>tom</u>, with (previously) novel properties: the element could only be detected phenotypically by its effect on eye morphology at 21 non-pleiotropic, non-dosage compensated, non-random loci. Because of the recovery of a spontaneous singed mutation, <u>sn⁹⁹</u>, in an ocular morphology (<u>Om</u>) mutant line derivative, <u>Om(1D)9g</u>, it was possible to investigate his speculation. <u>D. melanogaster</u> singed, <u>sn</u>, DNA probes were used to isolate and recover a <u>D. ananassae</u> singed gene. A comparison of the <u>sn⁹⁹</u> and wild type singed restriction map implicated a 6.5 kb insert as the element responsible for the mutator effect. A total of 186 recombinant lines from four X-linked <u>Om</u> loci were examined. 80 were <u>Om</u> and 106 were non-<u>Om</u>; in all instances an <u>in situ</u> hybridization signal, when probed with <u>sn⁹⁹</u> insert, was found at appropriate locations on the polytene chromosomes. Linkage was complete and showed that the <u>sn⁹⁹</u> insert was homologous to sequences localized at the sites of <u>Om</u> mutants. </p> <p> Preliminary analysis of several isolated clones of the <u>tom</u> element indicate a fairly conserved structure typical of copia-like elements. DNA sequence analysis of the <u>tom</u> at <u>sn⁹⁹</u> and <u>Om(1D)9g</u> showed direct repeats at the termini that is also characteristic of copia-like elements. On going southern blot studies of several <u>Om(1D)</u> mutations indicate that some are due to insertions of a single <u>tom</u>, others appear to be due to multiple copies and yet others show no obvious alterations in the immediate vicinity of <u>Om(1D)9g</u>. Further investigations of the <u>tom</u> elements and the <u>Om</u> mutations should provide insight into the mechanisms underlying this very unusual mutational process. </p>		

PROJECT DESCRIPTION

NATURE AND OBJECTIVES OF PROJECT: Hinton (1984) speculated that ocular morphology (Om) mutability in D. ananassae involved a transposable element whose target site of insertion was encoded in the control sequence found at eye morphology genes. Om mutability requires homozygosity of the ca;px stock X chromosome, or, in heterozygous females, the presence of either of two self-replicating extrachromosomal elements involved in male crossing over or mutability. Om mutability involves the spontaneous mutation at approximately 15 non-random loci, resulting in semidominant, non-dosage compensated, non-pleiotropic eye morphology mutations. While alleles of the same locus have similarities, they could not be unambiguously assigned to loci by phenotype alone. Om(1D)9, a particularly unstable allele, produced several derivatives, one of which, Om(1D)9g, was more extreme and was accompanied by a linked singed mutant, sn^{9g}.

The purpose of the present study was to establish the connection between Om mutability and sn^{9g}, i.e., to investigate whether sn^{9g} is the result of a mistaken Om event. The singed allele was chosen because it was from one of the very few loci mutated in Hinton's Om experiments that had been cloned in Drosophila. Once the causative transposable element, tom, was isolated, the further purpose was to study the structure of toms, their target sites and the nature of the Om sites in order to understand the mechanisms by which tom moves and causes unusual phenotypes at specific locations.

METHODS EMPLOYED: The Cloning of the Om element. DNA probes from the singed locus of D. melanogaster were used to clone the homologous region of D. ananassae. A comparison of sn^{9g} and wild type singed loci detected a 6.5 kb insert in the sn^{9g} locus. Genomic Southern blotting and in situ hybridization techniques were used to show that this insert was repetitive and dispersed. There was a preponderance of copies on the X chromosome and a survey of several stocks indicated that this element was absent from some stocks.

Recombinant Om lines from 4 Om stocks were manufactured, isolated and made homozygous for in situ hybridization with the 6.5 kb insert clone. Complete genetic linkage between the Om phenotype and in situ hybridization signal was demonstrated.

Several clones bearing insertions of the sequences homologous to the sn^{9g} were isolated, including that from Om(1D)9g. Preliminary restriction maps have been made and DNA sequence analysis has been carried out on these DNAs.

MAJOR FINDINGS AND PROPOSED COURSE: The 6.5 kb insert cloned from sn^{9g} was shown to be a member of a sequence family, tom, found at Om mutations in D. ananassae (another site). With the exception of the sn^{9g} mutation the Om mutator is unique in that it is phenotypically detected through its effect on eye morphology at a fixed number of sites.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In a comparatively short time the generally accepted view of a largely rigid immobile genome has been changed by the discovery and study of mobile genetic elements. The role of these elements in mutation, cancer, evolution and speciation is of

great interest and importance; therefore, a delineation of their properties is essential. Some of the hypothesized properties of the Om element are unprecedented since the phenotypic effects of the element are only detected at 21 non-random sites and only have an effect on eye morphology. The uniqueness of this mutator system may afford some new understanding of the biology of transposable elements and their impact on host organisms. Basic knowledge of this potential source of spontaneous mutation is fundamental to the understanding of genetic disease.

The preliminary analysis of insertions of sequences homologous to the 6.5 Kb insertion form sn⁹⁹ indicated that they form a conserved family, tom, with most of the typical copia-like characteristics. Incomplete Southern blot studies of several Om(1D) mutations identified several types of mutations: single insertions at or near the Om(1D)9 site, multiple insertions at the Om(1D)9 and several with no apparent alteration in the immediate vicinity of the Om(1D)9 site. Planned work includes the completion of the characterization of tom and its insertions especially at Om(1D). Analysis of Om suppressor mutations, Soms, is planned. The major effort will be to investigate the extrachromosomal, self-replicating factors that interact with the Om mutator.

PUBLICATIONS

Shrimpton, A.E. and Langley, C.H. A transposable element causes specific eye morphology mutations in Drosophila ananassae. (submitted to Genetics), 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61031-02 LG																				
PERIOD COVERED October 1, 1984 to September 30, 1985																						
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Molecular Population Genetics of Transposable Elements																						
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">C.H. Langley</td> <td style="width: 30%;">Research Geneticist</td> <td style="width: 10%;">LG, NIEHS</td> </tr> <tr> <td colspan="4" style="padding-top: 10px;">Others:</td> </tr> <tr> <td></td> <td>Elizabeth A. Montgomery</td> <td>Bio. Lab. Tech.</td> <td>LG, NIEHS</td> </tr> <tr> <td></td> <td>Clara S. Millis</td> <td>Guest Worker</td> <td>LG, NIEHS</td> </tr> <tr> <td></td> <td>Shiu L. Huang</td> <td>Guest Worker</td> <td>LG, NIEHS</td> </tr> </table>			PI:	C.H. Langley	Research Geneticist	LG, NIEHS	Others:					Elizabeth A. Montgomery	Bio. Lab. Tech.	LG, NIEHS		Clara S. Millis	Guest Worker	LG, NIEHS		Shiu L. Huang	Guest Worker	LG, NIEHS
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	Clara S. Millis	Guest Worker	LG, NIEHS																			
	Shiu L. Huang	Guest Worker	LG, NIEHS																			
COOPERATING UNITS (if any) Dr. Shiu L. Huang and Clara S. Millis Environment Health Research and Testing Inc. Research Triangle Park, N. C.																						
LAB/BRANCH Laboratory of Genetics																						
SECTION Eukaryotic Gene Structure Section																						
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																						
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:																				
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews													
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither																				
<input type="checkbox"/> (a1) Minors																						
<input type="checkbox"/> (a2) Interviews																						
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Study continued under Project Number Z01 ES 61035-01 LG entitled "Molecular characterization of spontaneous HGPRT mutations".																						

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61032-02 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure-function of mammalian lactate dehydrogenase isozymes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div style="width: 30%;">PI: Steven S.-L. Li</div> <div style="width: 35%;">Research Geneticist</div> <div style="width: 30%;">LG, NIEHS</div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div style="width: 30%;">Others: Farida S. Sharief</div> <div style="width: 35%;">Biologist</div> <div style="width: 30%;">LG, NIEHS</div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Genetics		
SECTION Eukaryotic Gene Structure Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (b) Human tissues </div> <div style="width: 30%;"> <input type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p style="margin-top: 10px;"> The complete primary structure of LDH-A₄ isozymes from human and mouse has been determined by sequence analyses of LDH-A cDNA and protein. The structural relationship of cancer-associated LDH-K to LDH-A isozyme has been illustrated. Partial amino acid sequences of LDH-B₄ isozymes from human and mouse have also been determined by amino acid sequencing. The amino acid sequences of 100% of the 330 residues from mouse testicular LDH-C₄ and 84% of rate LDH-C₄ have been determined. Sequence comparison among mammalian LDH isozymes clearly indicates that A₄ (muscle) and B₄ (heart) isozymes shows a closer evolutionary relationship to each other than either to the C₄ (testis) isozyme. Recently, LDH-A₄ isozymes have been found to be single-stranded DNA binding proteins which may play important roles in DNA replication, repair and recombination. The relationship of the protein structure to multiple functions as enzyme and as DNA binding protein will be further studied by <u>in vitro</u> directed mutagenesis. </p>		

PROJECT DESCRIPTION

PROBLEM: Structure and function of mammalian ssDNA-binding lactate dehydrogenase proteins.

OBJECTIVES: In mammals and birds three homotetrameric LDH isozymes A₄ (muscle), B₄ (heart) and C₄ (testis) possess distinct physical, catalytic and immunological properties. Recently, the ssDNA-binding protein from rat liver was found to be extremely homologous to pig LDH-A sequence. The structural relationship of the mammalian LDH proteins will be correlated with the ssDNA-binding and enzymatic activities.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: In human, the different clinical features of LDH-A or LDH-B deficiencies have been correlated with the impaired ATP production under anaerobic condition for LDH-A, but not LDH-B deficiency. The LDH-A₄ isozyme has also been shown to increase predominantly in many human cancer tissues. The LDH-A₄ isozyme along with enolase and phosphoglycerate mutase have been reported to be phosphorylated at tyrosine residue(s) in cells transformed by Rous sarcoma virus. The functional significance of these structural modifications will be investigated. The structural relationship of the LDH proteins to the ssDNA-binding and enzymatic activities will be investigated by the in vitro directed mutagenesis and post-translational modification.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: The primary structure of human and mouse LDH-A₄ isozyme has been determined by sequence analyses of the protein and cloned cDNA. The structural relationship of cancer-associated LDH-K to LDH-A isozyme has been illustrated. Partial amino acid sequences of human and mouse LDH-B₄ have also been obtained by direct protein sequencing. The structures of LDH-C₄ isozymes from mouse and rat testes have been established by amino acid sequencing and the space-filling models of the LDH-C₄ molecules have been constructed on a computer-graphic system.

This available information on primary and 3-D structures of mammalian LDH-A₄, B₄ and C₄ isozymes makes it possible to discuss their functional, immunological and evolutionary relationships. The different amino acid sequences at the LDH active center have been correlated with different kinetic properties of LDH-A₄, B₄ and C₄ isozymes. The amino acid sequence of the loop region of sperm-specific LDH-C₄ was shown to be quite different from those of somatic LDH-A₄ and B₄ isozymes, and it may account for its unique physical and chemical properties: thermal stability, broad substrate specificity and low turnover numbers. The immunological properties of mouse and rat LDH-C₄ have been compared with those of mouse LDH-A₄ and LDH-B₄ isozymes, and the potential antigenic determinants of LDH-C₄ isozymes have also been predicted on the basis of the hydrophilicity analysis of their amino acid sequences and three-dimensional protein structure. The LDH-C₄ isozyme is immunologically distinct from LDH-A₄ and LDH-B₄ isozymes, and the active immunization of animals

with LDH-C₄ autoantigen has lead to immunogenic impairment of fertility in females. Amino acid sequence comparison among vertebrate LDH isozymes clearly indicates that A₄ (muscle) and B₄ (heart) isozymes show a closer evolutionary relationship to each other than either to the C₄ (testis) isozyme.

FUTURE RESEARCH PLANS: 1. The primary structure of human and mouse LDH-B, and human LDH-C proteins will also be determined by sequence analyses of the proteins and cDNAs. The potential antigenic determinants of human LDH-C₄ isozyme will be analyzed and some of antigenic peptides may be synthesized chemically and tested for possible impairment of fertility.

2. The *in vitro* mutagenesis of LDH-A cDNA will be used to illustrate the structural relationships to ssDNA-binding and enzymatic activities of LDH proteins. The molecular nature of spontaneous and induced mutant LDH-A₄ proteins from mouse may also be determined.

3. The structural modification of LDH isozyme by oncogene src tyrosine kinase and its functional significance will be investigated. The dephosphorylation of tyrosine residue(s) by acid phosphatase will also be studied. The possible relationship between the LDH isozymes and oncogene ras proteins (H-ras, K-ras & N-ras) will be investigated.

PUBLICATIONS

Pan, Y.-C.E., Sharief, F.S., Okabe, M., Huang, S. and Li, S.S.-L.: Amino acid sequence studies on lactate dehydrogenase C₄ isozymes from mouse and rat testes. J. Biol. Chem. 258: 7005-7016, 1983.

Li, S.S.-L., Feldmann, R.J., Okabe, M., and Pan, Y.-C.E.: Molecular features and immunological properties of lactate dehydrogenase C₄ isozymes from mouse and rat testes. J. Biol. Chem. 258: 7017-7028, 1983.

Li, S.S.-L., Fitch, W.M., Pan, Y.-C.E., and Sharief, F.S.: Evolutionary relationships of vertebrate lactate dehydrogenase isozymes A₄ (muscle), B₄ (heart) and C₄ (testes). J. Biol. Chem. 258: 7029-7032, 1983.

Tsujibo, H., Tiano, H.F. and Li, S.S.-L.: Nucleotide sequences of the cDNA and an intronless pseudogene for human lactate dehydrogenase-A isozyme. Eur. J. Biochemistry 147: 9-15, 1985.

Li, S.S.-L., Tiano, H.F., Fukasawa, K., Yagi, K., Shimuzu, M., Sharief, F.S., Nakashima, Y., and Pan, Y.-C.E.: Protein structure and gene organization of mouse lactate dehydrogenase-A isozyme. Eur. J. Biochemistry (in press), 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61033-02 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Analysis of Genetic Variation in lens crystallin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Loren C. Skow Sen. Staff Fellow LG, NIEHS

Others: Maria E. Donner Fogarty Fellow LG, NIEHS
Shu-Mei Huang Research Associate LG, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to study the mechanisms responsible for heritable lens dysfunction resulting in anophthalmia or cataract formation in mice. To facilitate our studies of mouse eye mutants, we have been searching among inbred strains for genetic variation in lens crystallin genes using cDNA probes for α -, β - and γ -crystallins. Southern blot analysis of α A-crystallin sequences has detected eight restriction fragment size classes among 42 inbred strains of mice. The polymorphisms are due, in large part to insertions or deletions, in non-coding regions in or near the α A-crystallin gene. Gene mapping experiments have located the α A gene (designated Acry-1) to chromosome 17, very close to the mouse major histocompatibility region, H-2K. Striking linkage disequilibrium is observed between Acry-1 alleles and H-2 haplotypes among inbred strains, leading to the hypothesis that Acry-1 should be included as a component of the MHC. Similar experiments conducted on inbred strains of rats have confirmed the conservation of Acry-1 - MHC in this species. Conservation of other genes in the MHC region of mice and humans implies that the human Acry-1 gene is linked to HLA on chromosome 6.

Analysis of a β -crystallin sequence (Bcry-1) by 17 endonucleases has failed to detect restriction fragment polymorphisms among inbred strains. Analysis of DNA from mouse x hamster somatic cell hybrid clones located Bcry-1 to chromosome 11.

In contrast to the lack of variation in Bcry-1, γ -crystallin sequences (Gcry-1, -2, -3, -4) have demonstrated restriction polymorphism with all endonuclease so far employed. In gene mapping experiments, we have found no recombination between Gcry and a γ -crystallin protein variant (LEN-1) on chromosome 1 very near to the mutant gene, Elo, which produces anophthalmia in mice.

PROJECT DESCRIPTION

PROBLEM: The vertebrate eye is exquisitely sensitive to chemical and physical damage and is widely used as a test organ in genetic and somatic toxicology. Little is known about the normal development and biochemistry of the eye. Consequently it is difficult to assess the mechanisms involved in ocular toxicology. Numerous non-allelic mutations affecting eye development and function are maintained in mice and many of these mutations have been induced by chemical or physical mutagens. The purpose of this project is to use induced and naturally occurring genetic variation to investigate the processes controlling development and function of the eye.

OBJECTIVES:

1. Locate and determine the structural features of the α -, β - and γ -crystallin electrophoretic variants and restriction fragment polymorphisms in or near the genes encoding the crystallin proteins.
2. Conduct genetic and molecular analyses of mouse eye mutants with emphasis on complementation analysis and linkage studies.
3. Evaluate crystallin gene expression in eye mutants with emphasis on expression of γ -crystallin mRNA in the anophthalmic C3H/HeJ - Elo mouse.

ACCOMPLISHMENTS

1. The mouse α -crystallin gene (Acry-1) has been mapped to chromosome 17 by restriction fragment size polymorphisms. Analysis of recombinant inbred and congenic strains have located the gene between the glyoxalase (Glo-1) locus and H-2K about 1.4 cM from H-2K. Analysis of the Acry-1 variation by several restriction endonucleases revealed that the variation is due to insertions in the flanking regions.
2. The distribution of Acry-1 alleles and histocompatibility (H-2) haplotypes among inbred strains of mice indicates strong linkage disequilibrium between these loci. In collaboration with Drs. Dorothea Bennett, Memorial Sloan Kettering, Cancer Institute, and Joe Nadeau, The Jackson Laboratory, we have examined the Acry-1 locus in mice bearing t-haplotypes to evaluate the origin(s) and relationships of various t-haplotypes. Analysis of 17 different t-haplotypes revealed four alleles, three of which have not yet been observed among inbred strains or subspecies of Mus musculus. These data generally support the hypothesis that t-haplotypes are ancient and recombinationally isolated segments of the genome but indicate at least two origins of t-haplotypes, one of which must have occurred in the progenitor of modern Mus musculus.
3. Linkage of Acry-1 with H-2 in the mouse implies a conservation of this linkage group in mammals since genes within or flanking the MHC are syntenic in diverse species of mammals. In collaboration with Dr. T.J. Gill, University of Pittsburgh, we have analyzed Acry-1 restriction fragment polymorphisms in inbred strains of rats and confirmed that the Acry-1 - MHC linkage is maintained in this species.

4. Restriction endonuclease analysis of DNA from C3H/HeJ - Elo, an anophthalmic strain and the congenic normal strain C3H/HeJ has not detected any structural differences in the γ -crystallin gene sequences of Elo. These results suggest that Elo mice may be abnormal for γ -crystallin gene expression but that the structural genes remain essentially intact. Analysis of γ -crystallin by isoelectrofocusing revealed that lenses from Elo homozygotes are deficient in a specific γ -crystallin, LEN-1. Genetic recombination experiments have failed to separate Len-1 and Elo, suggesting that the failure of embryonic lens growth and subsequent normal eye development is a consequence of the absence of Len-1 gene expression.
5. Five crystallin fractions have been purified from mouse lens extracts and used to produce antisera in rabbits. Western blot analysis has revealed that LEN-2, a low molecular weight crystallin of the classical γ -crystallin component, shares antigenic determinants with β -crystallin, thereby confirming the conclusion, based on amino acid composition, that LEN-2 is a member of the β -crystallin family.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A knowledge of the mechanisms by which genotoxic substances interrupt normal developmental sequences is crucial to ascertain the risk associated with exposure to mutagenic and teratogenic compounds. The development and function of the vertebrate eye is exquisitely sensitive to perturbation by a variety of physical and chemical agents and therefore, presents an excellent system in which to investigate normal and aberrant developmental patterns. By using the numerous genetic mutants available in the mouse, we will be able to analyze developmental processes in eye formation and come to an understanding of how mutagens and teratogens disturb normal eye development.

RESEARCH PLANS FOR 1984-1985

The PI has accepted a University position. Projects will be terminated July 1, 1985.

PUBLICATIONS

Skow, L.C., Popp, R.A., and Bailiff, E.G.: A second polymorphic lens crystallin (Len-2) in the mouse. Genetics and biochemical analysis of LEN-1 and LEN-2. Biochemical Genetics 23:181-189, 1985.

Antonucci, T.K., von Diemling, O.H., Rosenblum, B.B., Skow, L.C., and Meisler, M.H.: Conserved linkage within a four centimorgan region of mouse chromosome 9 and human chromosome 11. Genetics 107:463-475, 1985.

Frater-Schröder, M., Prochazka, M., Haller, O., Arvert, F., Porck, H. J., Lundin, L.G., Skow, L.C., Hilkens, I., and Hilgers, J.: Localization of transcobalamin II (Tcn-2) on chromosome 11: Linkage to waved-2 (wa-2) and the hemoglobin e-chain Tocus (Hba). Biochemical Genetics 23:139-153, 1985.

Popp, R.A., Popp, D.M., Johnson, F.M., Skow, L.C., and Lewis, S.C.: Hematology of a murine beta thalassemia. A longitudinal study. Annals N.Y. Acad. Science (In Press) 1985.

Uiterdyk, H.G., Ponder, B.A.J., Festing, M.F.W., Hilgers, J., Skow, L.C., and vanNie, R. The gene controlling the binding sites of Dolichos biflorus agglutinin is on chromosome 11 of the mouse. Genetical Research (In Press), 1985.

Skow, L.C., Kunz, H.W., and Gill, T.J. Linkage of the locus encoding the A chain of alpha-crystallin (Acry-1) to the major histocompatibility complex in the rat. Immunogenetics (In Press), 1985.

Donner, M.E., Skow, L.C., Kunz, H.W., and Gill, T.J. Electrophoretic variation in lens low molecular weight crystallins from inbred strains of rats. Biochemical Genetics (In Press), 1985.

Lewis, S.E., Johnson, F.M., Skow, L.C., Popp, D.M., Barnett, C.B., and Popp, R.A. A mutation in the β -globin gene detected in the progeny of a female mouse treated with ethylnitrosourea. Proc. Natl. Acad. Science (In Press), 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61034-01 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Survey of Genetic Variation in Natural Populations of *Drosophila*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Kreitman Staff Fellow LG, NIEHS

Others: W. Quattlebaum Bio. Lab. Tech. LG, NIEHS
C.H. Langley Research Geneticist LG, NIEHS
Cynthia Newlin Biological Aid LG, NIEHS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

1.2

OTHER:

1.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The evolutionary histories of two genetic loci in *Drosophila* species are being examined by studying genetic variation at the DNA level in natural populations. A methodology has been developed to identify classes of haplotypes in large samples (100 lines or greater). The method involves probing filters containing an image of genomic DNA cut with four-cutter restriction enzymes and run under denaturing conditions on DNA sequencing-type gels. Approximately 20% of all nucleotide polymorphisms and all length polymorphisms can be identified within the probed region. This approach is being applied to samples of approximately 100 lines from each of several populations of *D. melanogaster* and a single population of its sibling species, *D. simulans*. Two loci are under investigation - Alcohol dehydrogenase and White.

PROJECT DESCRIPTION

PROBLEM: A large amount of nucleotide and length polymorphism has been identified at the alcohol dehydrogenase (Adh) locus in D. melanogaster but little is known about how this species-wide variation is distributed within natural populations and between species. Nor is it known whether the description of variation at the Adh locus is representative of other loci within the genome.

OBJECTIVES: The objective is to understand the evolutionary structure of natural populations of Drosophila by studying how nucleotide polymorphisms within the species are distributed within and between natural populations and species.

EXPERIMENTAL APPROACHES AND SCIENTIFIC JUSTIFICATION: Previous molecular techniques (Southern blotting and DNA sequencing) have lacked either sufficient resolution (blotting) or power (sequencing) to study the structure of natural populations at the DNA level. The technique being employed here is sufficient to identify essentially all haplotypic variation at structural loci. This allows a stratified approach to studying variation in natural populations: first the identification of haplotypes and description of their evolutionary relationships by four-cutter analysis and second, the detailed examination of specific haplotype by direct cloning and DNA sequencing.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: Approximately 200 isofemale lines of a Raleigh, NC population of D. simulans have been inbred for 10 generations by sib-mating to provide homozygous lines for the analysis. Similarly, approximately 150 X-chromosomes and 150 second chromosomes have been isogenized from a Raleigh, NC collection of D. melanogaster and genomic DNA has been prepared from these lines. The alcohol dehydrogenase locus has been cloned from D. simulans and probes have been prepared.

PLANS FOR SUBSEQUENT YEARS: Genomic DNA will be prepared from the above lines as well as from lines collected from other populations of the two species. Four-cutter analysis will be performed and DNA sequencing studies of some of the genes will be initiated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The nature of mutational variation in natural populations is essential to an understanding of genetic disease. Drosophila offers a model system for studying this variation.

PUBLICATIONS

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61035-01 LG												
PERIOD COVERED October 1, 1984 to September 30, 1985														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Molecular characterization of spontaneous HGPRT mutations														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: C.H. Langley</td> <td style="width: 33%;">Research Geneticist</td> <td style="width: 33%;">LG, NIEHS</td> </tr> <tr> <td>Others: Elizabeth A. Montgomery</td> <td>Bio. Lab. Tech.</td> <td>LG, NIEHS</td> </tr> <tr> <td>Clara S. Millis</td> <td>Guest Worker</td> <td>LG, NIEHS</td> </tr> <tr> <td>Shiu L. Huang</td> <td>Guest Worker</td> <td>LG, NIEHS</td> </tr> </table>			PI: C.H. Langley	Research Geneticist	LG, NIEHS	Others: Elizabeth A. Montgomery	Bio. Lab. Tech.	LG, NIEHS	Clara S. Millis	Guest Worker	LG, NIEHS	Shiu L. Huang	Guest Worker	LG, NIEHS
PI: C.H. Langley	Research Geneticist	LG, NIEHS												
Others: Elizabeth A. Montgomery	Bio. Lab. Tech.	LG, NIEHS												
Clara S. Millis	Guest Worker	LG, NIEHS												
Shiu L. Huang	Guest Worker	LG, NIEHS												
COOPERATING UNITS (if any) Dr. Shiu L. Huang and Clara S. Millis Environment Health Research and Testing Inc. Research Triangle Park, N. C.														
LAB/BRANCH Laboratory of Genetics														
SECTION Eukaryotic Gene Structure Section														
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709														
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 0.5	OTHER: 1.0												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The types of gene structural changes causing deficiency of hypoxanthine guanine phosphoribosyl transferase (HGPRT) activity in spontaneous mutations is being examined in cultured human fibroblasts. The deficiency of this enzyme activity causes a human disease (Lesch-Nyhan Syndrome). The restriction enzyme cleavage patterns of HGPRT gene sequences in mutant lines is being analyzed. The work is presently focused on obtaining a large number of independent spontaneous mutants that existed in new born baby's foreskins. Forty independent mutants have been isolated from different normal newborns. The mutant cells were grown to large numbers. Portions of cultured cells were frozen in liquid nitrogen for cytogenetic and enzymology studies at a future time and portions of cultured cells were frozen for DNA extraction. Southern blot analysis is now in progress to assess the possible involvement of DNA rearrangements in spontaneous mutation.</p> <p>(Continuation of Project No. Z01 ES 61031-02 LG)</p>														

PROJECT DESCRIPTION

PROBLEM: It is fundamental to the understanding of the basic process of spontaneous mutagenesis to collect and characterize mutations in well studied loci. Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is one of the best studied loci in cultured human cells. Germline mutations at this locus cause the Lesch-Nyhan syndrome. Some of these have been studied in other laboratories and a few have been shown to be associated with deletions of part or all of the locus (approximately 34 Kb). The problem this project addresses is the nature of spontaneous mutations that arise in cultured human cells.

OBJECTIVES: This project is aimed at characterizing a collection of independent spontaneous HGPRT mutations that arose in cultured diploid human fibroblasts in order to gain some insight into the qualitative nature of spontaneous mutagenesis in somatic cells.

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: The recent cloning of the human HGPRT structural gene sequences has now made it possible to carry out the structural analysis of mutant human HGPRT genes. Identification of mutation in cultured cells newborns previously had been limited to enzyme assays. The enzyme activity of mutants ranges from none to full activity. It is important to understand the relationship between the residual enzyme activity of the gene products and the structure of genes, at the molecular level. Primary human cell cultures were established from neonatal foreskins. To assure the independent origin of mutants, one mutant from each primary culture was used for molecular analysis. Mutant cells form colonies in 6-TG medium. Through several culture passages, the mutant cells grow to large numbers. To determine the nature of mutations at the molecular level, DNA was purified from each of the mutants and digested with restriction endonucleases. After fractionation by agarose gel electrophoresis, HGPRT-containing sequences could be visualized by Southern blotting and hybridization with a non-repetitive fragment of the HPRT clone labeled to high specific activity by nick-translation.

RECENT ACCOMPLISHMENTS: Since normal human cells senesce, mutant cells derived from non-primary culture cease to grow before a number of cells sufficient for analysis can be obtained. Primary cultures, therefore, offer several advantages. 1. Mutants cells are young and can yield enough DNA for analyses. 2. Mutants isolated from foreskins are preexisting ones and represent mutations in vivo. 3. Mutants from different individuals are of independent origin. Fifty foreskins have been collected, from which primary cultures have been established. Mutants from 40 primary cultures have been isolated and stored in liquid nitrogen. Mutant cell lines have been grown to $5-6 \times 10^6$ cells for DNA extraction.

PLANS FOR SUBSEQUENT YEAR: We are presently in the process of isolating and purifying DNA from mutant cell lines, and refining molecular techniques for human DNA analysis. Experiments will be carried out in next two years to analyze the genetic structure of mutant DNA and to measure the residual enzyme activity of mutants.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The nature of spontaneous mutations is an important component of a fundamental understanding of genetic disease. Insertions are a major part of spontaneous mutation in several experimental organisms. It is important to determine whether this is also the case in human cells (both germline and somatic).

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61036-01 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Naturally occurring DNA Sequence Variation in <i>Drosophila melanogaster</i>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Charles F. Aquadro Staff Fellow LG, NIEHS Others: Charles H. Langley Research Geneticist LG, NIEHS		
COOPERATING UNITS (if any) Dr. C. Laurie-Ahlberg, Associate Professor of Genetics North Carolina State University, Raleigh, North Carolina		
LAB/BRANCH Laboratory of Genetics		
SECTION Eukaryotic Gene Structure Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The objective of this project is to examine the patterns and levels of naturally occurring DNA sequence variation in a variety of gene regions of <i>D. melanogaster</i>. Of particular interest is the effect of sequence alterations within and flanking the gene encoding the enzyme dopa decarboxylase (Ddc). No clear patterns between sequence variants and Ddc expression were observed, despite two-fold variation in Ddc enzyme activity among the 46 lines examined. Located adjacent to the 30 - 40 kilobases of DNA sequence containing a dense cluster of eight lethal-mutable genes (including Ddc) is an approximately 40 kilobase region in which, at most, one gene appears to be located. If mutations occur randomly throughout the genome, yet persist for any length of time only in regions where they produce little or no deleterious effect, we should see less variation in the dense cluster of genes than in the adjacent 40 kb region. Examination of DNA sequence variation by restriction mapping in this 80 kilobase region has revealed the opposite pattern. While we cannot rule out the selective maintenance of variation in the gene cluster, these results raise the possibility of increased insertion/deletion mutational activity in transcriptionally active regions although unknown constraints may exist in the nonvariable region. We have also examined variations in several other regions of the <i>Drosophila</i> genome including the genes for white, notch, rudimentary, amylase and an amylase pseudogene. Strong support has been obtained for the hypothesis that insertions/deletions, and transposable element insertions in particular, are deleterious. </p>		

PROJECT DESCRIPTION

PROBLEM: Our understanding of the frequency, nature of, and significance of naturally occurring DNA sequence variation is in its infancy. Little is known of the extent of the generality of transposable element insertion site specificity such as that seen in our examination of the alcohol dehydrogenase region of Drosophila melanogaster. In addition, we have little knowledge of the extent to which natural selection maintains DNA sequence variation and nonrandom associations of sequence variants at linked genes. All of this information is important if we are to understand the significance of the sequence diversity apparently present in most organisms, including humans, and if we are to be able to assess the long-term significance of mutational damage.

OBJECTIVES: The objectives are to determine (1) What levels of DNA sequence variation occur normally. (2) Where, relative to structural genes or other regions of defined function, these sequence alterations occur. (3) Whether transcriptionally active regions are more variable than nontranscribed regions. (4) The extent and origin of nonrandom associations among DNA sequence variants along the chromosome.

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: Information concerning the extent, location and significance of naturally occurring DNA sequence variation is central to the assessment of the significance of mutagen exposure. In addition, studies of apparently normal individuals provides important information concerning the nature and size of the "target" for mutagenesis and the types of variation that can and cannot be tolerated. A population genetics approach is particularly valuable for these types of problems since effects of an extremely subtle, yet significant, level of natural selection can often be detected since we effectively examine the variant's significance over thousands or hundreds of thousands of generations.

We have chosen to study Drosophila melanogaster from natural populations and to focus on the regions around the structural genes for dopa decarboxylase, notch, white, rudimentary, amylase and an amylase pseudogene for several reasons. Foremost is the fact that these are well studied regions in terms of genetics, biochemistry and molecular genetics. For example, the dopa decarboxylase structural gene, Ddc, located on chromosome II, catalyzes the decarboxylation of dopa to dopamine and 5-hydroxytryptophan to serotonin. Enzymatic activity is required for cuticle hardening, female fertility, tissue specificity and proper functioning of the central nervous system. Ddc is located in the middle of a large dense cluster of 17 genes and thus provides us an ideal opportunity to examine sequence variation in a functionally related gene cluster. In addition, two quite different regions appear to be juxtaposed: one region containing Ddc and at least 6 to 7 additional genes (identified as lethals and regions encoding messenger RNAs), some of which appear to be functionally related and packed within roughly 30 kb of DNA sequence; and a second region immediately adjacent that contains at most one identified gene in well over 40 kb of sequence. Importantly, over 100 kb of this entire Ddc region had been cloned by J. Hirsh

who kindly has provided us with the clones which we can use to probe the entire region, including the transcribed and nontranscribed portions. Studies of this magnitude are generally not possible in humans due to the large number of small repeat sequences scattered throughout the mammalian genome making it difficult to obtain probes to completely assay large regions of the mammalian genome in the same way.

Approximately 80 lines containing genetically isolated second chromosomes of D. melanogaster were reared and nuclear DNA isolated. Restriction maps were constructed for 20 to 80 kb regions around each gene and compared for evidence of nucleotide substitutions and insertion/deletion variation. Gene expression at the Ddc gene was assayed by examining enzymatic activity. We were not able to assay gene expression at the other genes surrounding Ddc since they are at present known only by their lethal phenotype and presence of messenger RNA (the precise limits of which have not yet been defined). Amylase expression was assayed by enzymatic activity.

RECENT ACCOMPLISHMENTS: Forty-six second-chromosome lines of Drosophila melanogaster isolated from five natural populations were surveyed for restriction map variation in an 80 kb region surrounding the gene encoding dopa decarboxylase (Ddc). Eighty-four restriction sites were scored, 24 of which were polymorphic. While the polymorphic sites occur throughout the 80 kb region, those that are highly variable are clustered within a 40 kb region containing Ddc and a dense cluster of functionally related lethal genes. Five large (1.5-5 kb) inserts, one small insert (250 bp) and four small (100-200 bp) deletions were observed to be similarly localized to the 40 kb region. Surprisingly, no insertion/deletion variation was observed in the 40 kb region that appears to contain at most one gene. Significant nonrandom associations were observed among restriction sites in approximately the same 40 kb region but drop off 5' to the gene cluster. That all measures of variation show a marked decrease at virtually the same location (the break between the functionally related gene cluster and the genetically sparse region) suggests that the patterns are not due simply to chance and may reflect selection for favored combinations of alleles in this cluster of genes associated with female fertility and cuticle formation.

We have also assayed adult DDC activity in these lines. Surprisingly, two lines with 5 and 1.5 kb inserts within an intron of and at the 5' end of Ddc, respectively, show completely normal adult activities. Unlike our results for the alcohol dehydrogenase region of these same lines, no consistent pattern of association between level of Ddc activity and restriction site haplotype is apparent, although two-fold variation in activity among lines is observed within this sample of 46 lines.

Similar results have been obtained for the nearly 200 additional kilobases assayed around the loci white, rudimentary, Notch, amylase and amylase pseudogene. In no case have transposable elements been observed at other than rare frequencies at any one site, a result consistent with their being slightly deleterious. The primary mechanism of selection is probably on total copy number of the element in the genome rather than on individual insertions. This numbers of any one element in the genome and the resultant increased frequency of chromosome rearrangement (in many cases related to recombination among elements in nonhomologous sites in the genome).

PLANS FOR SUBSEQUENT YEAR: Project terminates with departure of Dr. Charles F. Aquadro in August, 1985.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The study of naturally occurring DNA sequence variation provides information on background levels of variation, information vital to the assessment of the significance of mutagen exposure. The results also contribute significantly to determining the "target" for mutagenesis and the types of variation that can and cannot be tolerated.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61037-01 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanism of DNA Replication in Eucaryotes: I. Yeast as a Model System		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	A. Sugino	Visiting Scientist LG NIEHS
Others:	R. K. Hamatake	Staff Fellow LG NIEHS
	A. Sakai	Visiting Fellow LG NIEHS
	T. Chow	Visiting Associate CBTP NIEHS
	P. S. Alexander	Biologist LG NIEHS
	A. B. Clark	Biologist LG NIEHS
	R. Desai	Stay-In-Schooler LG NIEHS
	T. Sugino	Guest Worker LG NIEHS
COOPERATING UNITS (if any) Lucy M. S. Chang, Professor and Chairperson, Dept. of Biochemistry, The Uniformed Service University of Health Sciences Bethesda, MD 20705		
LAB/BRANCH Laboratory of Genetics		
SECTION Mutagenesis Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.85	2.1	2.75
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> An <i>in vitro</i> DNA replication system using yeast 2-μm and ARS plasmid DNA has been developed as a model to investigate the mechanism of DNA replication in eucaryotes. To elucidate the mechanisms of initiation, elongation and termination of DNA replication, this system has been further characterized. Recent biochemical fractionation and reconstitution experiments permitted the purification and characterization of yeast DNA primase, (60,000 daltons), single-stranded DNA binding protein, (38,000 daltons), DNA-dependent ATPase III (63,000 daltons) and DNA polymerase I (140,000 daltons). Furthermore, RNase Hs (21,000, 48,000 and 68,000 daltons) and two other single-stranded DNA binding proteins (14,000 and 20,000 daltons) which might participate in DNA replication have been purified to homogeneity by following their enzymatic activities. To prove that these proteins are required for yeast DNA replication, antibodies have been raised against each and their genes have been identified and cloned from λgt11 yeast genomic library using the antibodies. Currently, their nucleotide sequences are being determined. In order to permit identification and isolation of other DNA replication proteins, new temperature-sensitive DNA replication mutants of yeast have been isolated and characterized genetically; some of these mutant genes have been identified and cloned from the yeast genomic library. </p>		

Research Project

Problem: Our knowledge of the mechanism of DNA replication in procaryotes advanced tremendously after in vitro replication systems of small viral DNA were developed and various DNA replication mutants were isolated. Their complexity and the lack of good genetics, on the other hand, have retarded the understanding of eucaryotic DNA replication. Although the fundamental replication processes would be expected to be similar to those of prokaryotes, a more detailed analysis of DNA replication is important for a better understanding of DNA replication and ultimately for an understanding of DNA repair and mutagenesis in eucaryotes.

The yeast Saccharomyces cerevisiae, has many advantages as a model system for the study of eucaryotic DNA replication. It is simple, has been extensively characterized genetically, has a good DNA transformation system and is easily obtained in large amounts for biochemical characterization. Furthermore, most yeast strains contain an extrachromosomal element, 2- μ m plasmid DNA, whose replication is controlled by the same mechanism as is chromosomal DNA. The cloned chromosomal sequences (ARs) which support autonomous DNA replication in yeast cells have been isolated. Finally, an in vitro replication system of 2- μ m and ARS plasmid DNAs has been developed.

Objectives of Research Project: The objectives of this project are (1) to further characterize our in vitro replication system using yeast 2- μ m and ARS plasmid DNAs to uncover the mechanisms of initiation, elongation and termination of DNA synthesis in eucaryotes; (2) to fractionate and reconstitute the in vitro replication system to identify various DNA replication components; (3) to determine whether the identified and purified components are essential for in vivo DNA replication, to which end their genes will be cloned, mutagenized, integrated into the chromosome and the wild-type genes replaced by the mutagenized genes; (4) using a newly isolated and characterized set of yeast mutants which are temperature-sensitive for DNA synthesis to identify and isolate other replication components; and (5) to ease the preparation and characterization of the components, the proteins will be overproduced in either E. coli or yeast by recombinant DNA technology.

Experimental Approach and Scientific Justification: In previous years, a DNA primase and the 38,000 dalton single-stranded DNA binding protein (ySSB) have been isolated and purified to homogeneity from yeast crude extract using our in vitro replication system with 2- μ m and ARS plasmid DNAs. This approach promises to identify and purify other DNA replication components as well. Although we have been able to identify such components using the in vitro replication system, it is not certain that these are in fact necessary for in vivo DNA synthesis. To prove their essentiality, antibodies are raised against homogeneous preparations of the components. Using the antibodies as probes, the gene for each protein is identified and cloned from a λ gt11 expression-vector yeast genomic DNA library. Then, the isolated gene is disrupted by inserting a foreign DNA fragment or by deleting a part of the gene, and is integrated into the chromosome to replace the wild-type gene. Finally, cell viability is tested after sporulation and dissection of the spores.

In the past, few DNA replication mutants have been isolated in yeast, although its DNA replication is more complex than is bacterial DNA replication. Therefore, it is anticipated that many more DNA replication mutants can be isolated. After EMS mutagenesis, more than 100 new temperature-sensitive DNA replication mutants have been collected. These fall into 30 complementation groups, including three of the previously identified DNA replication mutants. These mutants are very useful to identify and purify the DNA replication components using the in vitro replication system.

Recent Accomplishments and Significance to Biomedical Research: A yeast DNA primase and a single-stranded DNA binding protein have been isolated and purified to homogeneity by using our in vitro replication system. The same approach has been extended and an additional DNA replication protein has been identified and purified. This is a new DNA-dependent ATPase which stimulates yeast DNA polymerase I and has DNA-unwinding activity. This ATPase activity is missing in the radiation-sensitive mutant rad3, strongly suggesting that RAD3 is a structural gene for the DNA-dependent ATPase. By biochemical activity assay, two additional single-stranded DNA binding proteins (14,000 and 20,000 daltons), three different RNase Hs (21,000, 48,000 and 68,000 daltons), DNA polymerases I and II (140,000 and 90,000 daltons), and a DNA-dependent ATPase (68,000 daltons) previously described by others have all been purified to homogeneity from yeast crude extract. Antibodies have been raised against each protein. Using the antibodies as probes, the genes for three different single-stranded DNA binding proteins, two RNase Hs, DNA dependent ATPases and DNA polymerase I have been identified and cloned from a λ gt11 yeast DNA library. Subcloning to reduce the size of the DNA fragment which codes for each protein, and nucleotide sequencing of each gene, are in progress. In the meantime, the cloned genes have been disrupted by inserting the yeast URA3 gene, integrating into the yeast chromosome by homologous recombination to replace the wild-type gene by the disrupted gene; it will then be determined whether or not the gene is essential for DNA replication.

The above antibody technique does not necessarily identify the gene for the purified protein in which we are interested. We have therefore been using alternative approaches. One is to determine the amino acid sequence of the purified protein and to synthesize oligonucleotides predicted from the amino acid sequence. These oligonucleotides will then be labeled with ^{32}P and used as probes to identify and clone the genes.

Thirty different complementation groups of DNA replication mutants have been characterized. Some of their mutations have been mapped on the yeast chromosome and the corresponding genes have been cloned by complementation.

By analogy to procaryotes, it is reasonable to imagine that DNA replication proteins form a complex (replisome) which can be isolated. Therefore, new DNA replication proteins have been identified and partially purified using the DNA polymerase I-antibody column. These include DNA topoisomerase II, RNase H, single-stranded DNA binding protein, DNA primase and yet other unidentified proteins.

Plans for Future:

For the next two or three years, we are going to continue the characterization, fractionation and reconstitution of our in vitro replication system using yeast 2- μ m plasmid DNA, since it has been proven that this approach is very useful and successful for identifying and purifying various DNA replication components and for understanding the mechanism of DNA replication in yeast. Furthermore, the gene cloning for each component, using λ gt11 expression yeast genomic DNA library, has been so successful thus far that it suggests future success for gene clonings, if the antibody for the specific protein is available. It is anticipated that several DNA replication components will be isolated, their genes will be cloned and their essentiality for DNA replication in vivo will be determined. After establishing which purified components are essential for DNA replication in vivo, we will try to overproduce the components in either E. coli or yeast cells.

Publications:

Sugino, A., Sakai, A., Wilson-Coleman, F.E., Arendes, J. and Kim, K.-C.: In vitro reconstitution of yeast 2- μ m plasmid DNA replication. Mechanisms of DNA Replication and Recombination (ed. Cozzarelli, N.R., Alan Liss, Inc., New York) pp. 527-552, 1983.

Resnick, M.A., Sugino, A., Nitiss, J. and Chow, T.: DNA polymerases, deoxyribonucleases, and recombination during meiosis in Saccharomyces cerevisiae. Mol. Cell. Biol. 4, 2811-2817, 1984.

Wilson, F.E. and Sugino, A.: Purification of a DNA primase activity from the yeast Saccharomyces cerevisiae: primase can be separated from DNA polymerase I. J. Biol. Chem. 260: (in press), 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 61038-01 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of DNA Replication in Eucaryotes: II. SV40 as a Model System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Sugino	Visiting Scientist	LG	NIEHS
	P. J. de Jong	Visiting Fellow	LG	NIEHS

Others:	P. Carl	Guest Worker	LG	NIEHS
	R. Symanski	Summer Aid	LG	NIEHS

COOPERATING UNITS (if any)

Yung-Chi Cheng, Professor
Dept. of Pharmacology, Univ. of North Carolina
Chapel Hill, NC 27514

LAB/BRANCH

Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

1.45

PROFESSIONAL:

1.2

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

DNA topoisomerases I and II, DNA primase, DNA-dependent ATPase and RNase H activities, all of which are expected to participate in DNA replication, have been purified to near homogeneity from human cell lines and calf thymus and monoclonal and polyclonal antibodies have been raised against each proteins. To determine whether these proteins are required for eucaryotic DNA replication, the soluble in vitro replication system of SV40 DNA (which mimics the in vivo system) has been established and used to test whether the antibodies inhibit in vitro SV40 DNA replication. This system makes possible not only the identification and purification of various DNA replication proteins but also the identification of other factors which maintain high fidelity of DNA synthesis. For measuring the fidelity of the in vitro SV40 replication system, chimeric M13mp2 phages have been constructed which consist of an SV40 DNA replication origin and the E. coli lacZ gene.

Research Project

Problem: The complex events that occur during the replication of eucaryotic chromosomes are not yet well understood. By analogy with recent findings in prokaryotic systems, it is expected that much of the basic information about chromosomal replication can be obtained by studying the replication of viruses and other extrachromosomal elements. Furthermore, it is anticipated that rapid progress in this area will require a detailed analysis of an in vitro system which faithfully carries out the replication of such genomes. Recently, an in vitro replication system of SV40 viral DNA has been developed by T. Kelly's group. This system mimics most aspects of in vivo replication. Therefore, the detail analysis of this system promises to uncover the mechanism of eucaryotic DNA replication.

Objectives: The objectives of this project are twofold. The first is to determine which the various purified enzymes are required for in vitro replication of SV40 DNA using the antibodies. Once it is proven that some of the proteins are required, the corresponding genes will be cloned from either a λ gt11 cDNA library or a genomic library. The second objective is to study the fidelity of the SV40 in vitro replication system and compare this fidelity with that of DNA synthesis catalyzed by purified DNA polymerases. If significant differences exist between in vitro SV40 replication and the purified DNA polymerase reaction, the replication system will be fractionated and the factors which contribute in higher fidelity will be isolated and characterized.

Experimental Approach and Scientific Justification: It is a difficult and time-consuming process to obtain temperature-sensitive DNA replication mutants from higher eucaryotes. Therefore, various enzymatic activities which are expected to participate in DNA replication will be purified extensively from human cell lines and calf thymus. Then, either polyclonal or monoclonal antibodies will be raised against these enzymes. The enzymatic activities chosen are DNA topoisomerases, DNA primase, DNA-dependent ATPase, DNA helicase, RNase H, and single-stranded DNA binding proteins. We will determine which purified enzymes are required for DNA replication using the SV40 in vitro replication system and the antibodies. If some antibodies inhibit in vitro DNA replication, the interpretation of the result will be simple. However, it is also possible that the antibodies do not inhibit in vitro DNA replication. One possibility is that SV40 DNA replication does not require such an enzyme while chromosomal DNA replication does. The other possibility is that an enzyme joins in a complex and thereby becomes insensitive to antibody. Although the first possibility is not yet easily tested, the latter is testable by the biochemical fractionation and reconstitution of the in vitro SV40 replication activity.

The in vitro replication system of SV40 DNA may contain almost all components necessary to maintain the high fidelity of DNA synthesis observed in vivo. To study the fidelity of the in vitro replication activity of SV40, chimeric M13mp2-SV40ori bacteriophages will be constructed. These allow the in vitro system to initiate DNA synthesis at the SV40 DNA replication origin and to extend DNA strands, thus copying the E. coli lacZ gene which is a target site for a mutation assay (see T. Kunkel's report). Once differences can be observed between in vitro replication and the purified DNA polymerase alpha, the in vitro

activity will be fractionated and fractions which increase the fidelity of the DNA polymerase alpha reaction will be identified and purified.

Recent Accomplishments and Significance to Biomedical Research: DNA topoisomerases I and II, DNA primase, DNA dependent ATPase, and RNase H have been purified to homogeneity from human cell lines and calf thymus and antibodies have been raised against each. To ask whether or not these proteins are needed for eucaryotic DNA replication, the in vitro replication system of SV40, developed originally by T. Kelly's group, has been established. While characterizing DNA topoisomerases from human cell lines, a new activity associated with a type I topoisomerase has been identified. This activity introduces positive supercoils into a fully relaxed, covalently closed double-stranded DNA without any nucleotide cofactor. We named this activity "reverse gyrase" since the supercoils generated by the enzyme are always positive, instead of the negative supercoils generated by bacterial DNA gyrases. This activity might regulate gene expression in higher eucaryotes.

In order to study the fidelity of in vitro replication of SV40 DNA and to compare it with that of the purified DNA polymerases, chemeric M13mp2 derivatives have been constructed which consist of an SV40 DNA replication origin and the E. coli lacZ gene.

Plans for Future:

If some of the purified proteins are required for in vitro SV40 DNA replication, the genes for the proteins will be identified and cloned from a λ gt11 human cDNA library. The regulation of each gene's expression will then be studied using the cloned genes.

The fidelity study using the SV40 in vitro system was initiated this year and will continue for the next two or three years to identify and purify the factors which increase the fidelity of purified DNA polymerases.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61039-01 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanism of DNA Recombination and Repair in Yeast <i>Saccharomyces cerevisiae</i>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	A. Sugino	Visiting Scientist LG NIEHS
Others:	F. W. Coleman	Senior Staff Fellow LG NIEHS
	B.-Y. Ryu	Guest Worker LG NIEHS
	T. Sugino	Guest Worker LG NIEHS
	T. Chow	Visiting Associate CBTP NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Genetics		
SECTION Mutagenesis Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.1	0.5
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Proteins binding to single-stranded DNA are expected to participate in DNA recombination and repair as well as DNA replication. Thus, three different single-stranded-DNA-binding proteins have been purified from the yeast <i>Saccharomyces cerevisiae</i> and antibodies have been raised against them. Using the antibodies as probes, their genes have been identified and cloned from a λ gt11 yeast DNA library. Deletions of these genes were then constructed, the wild-type genes were replaced by the disrupted genes, and the resulting phenotypes were studied. The RAD52 gene product is required for DNA recombination and repair in yeast. The gene has been cloned and its nucleotide sequence determined by other groups. However, this important gene product has not yet identified and purified. By aid of a computer we identified several possible antigenic regions in the RAD52 gene. The oligopeptides covering the antigenic regions were chemically synthesized and conjugated to BSA and antibodies were raised against the conjugates. In addition, several fusion plasmids of the RAD52 gene and either the λ pL promoter or the yeast α -mating type pheromon leader sequence or the yeast ADH promotor will be constructed in order to overproduce RAD52 protein in <i>E. coli</i> and yeast. Finally, an <i>in vitro</i> DNA recombination system yeast δ - δ sequences has been developed. This system requires ATP, $MgCl_2$, and super-coiled plasmid DNA containing at least two delta sequences; it generates a double-strand break near or at the one of delta sequences but does not cleave plasmid DNA lacking delta sequences.		

Research Project:

Problem: The yeast *Saccharomyces cerevisiae* has many advantages for studying DNA recombination and repair in eucaryotes. It has excellent genetics, is a very simple organism, and is easily prepared in large amounts for biochemical analysis. Furthermore, it has a good transformation system and it is very easy to construct mutants by in vitro mutagenesis. A large number of radiation, UV-, and some mutagen-sensitive mutants have been isolated and mapped on the yeast chromosome. However, very little is known about what kind of enzymes participate in DNA recombination and repair processes in yeast. Therefore, this project focuses on identification and purification of some proteins which are required for recombination and repair.

Objectives of Research Project: (1) An in vitro DNA recombination system which mimics the in vivo reaction will be developed from yeast crude extracts. (2) It will then be used for identifying and purifying the components necessary for the DNA recombination reaction. (3) By analogy to procaryotic systems, it is expected that DNA helicase, *E. coli* recA-like protein, nucleases, DNA topoisomerases, DNA ligase, and single-stranded DNA binding proteins are needed for recombination and repair in yeast. These activities will be purified and their genes will be isolated. (4) The isolated genes will be disrupted by insertion of DNA fragments and integrated into the chromosome to replace the wild-type gene; the resulting phenotype will then be studied. (5) Although several RAD genes have been cloned and their nucleotide sequences have been determined, few gene products has yet been isolated and purified. Therefore, we will try to identify and purify some of the gene products produced in this project, using oligopeptide-directed antibodies.

Experimental Approach and Scientific Justification: In order to develop an in vitro DNA recombination system, we have slightly modified our in vitro replication system based on 2- μ m and ARS plasmid DNAs. Crude extracts were made from either exponentially growing mitotic cells or synchronized meiotic cells in a manner similar to that used for the crude extract for the in vitro DNA replication system. The crude extracts were incubated with buffer, magnesium chloride, DTT, ATP and supertwisted plasmid DNA consisting of yeast delta sequences. Recombination products produced in vitro were analyzed by agarose gel electrophoresis. Delta sequences were chosen because six of them are located in the cloned sup4 sequence of yeast and the frequency of in vivo recombination among these delta sequences is at least 100 times higher than among normal genes. Also, this recombination requires the RAD52 gene product, as in the general recombination reaction. If such an in vitro recombination system can be developed, fractionation and reconstitution of the system will be employed to identify and purify various recombination proteins and factors.

Several proteins which might be participate in DNA recombination and DNA repair will be purified from yeast using assays of biochemical activity: single-stranded DNA binding proteins, DNA topoisomerases, DNA helicases, nucleases, and *E. coli* recA-like proteins. Antibodies will be raised against the purified proteins and the genes for each will be screened from a λ gt11 yeast library using the antibodies. Also, we will test whether or not the in vitro recombination system is inhibited by the antibodies.

In order to identify RAD52 and RAD3 gene products, oligopeptide-directed antibody has been raised. This method involves computer analysis of the nucleotide sequences to predict possible antigenic regions of the predicted gene product. The oligopeptides covering the candidate antigenic regions have been chemically synthesized and covalently linked to bovine serum albumin. Then the hybrid protein will be injected into rabbits to raise antibodies. These antibodies should specifically interact with the intact gene product and should be useful tools to identify and purify the gene products.

The second approach is to construct fusions between the yeast mating type alpha pheromone leader peptide sequence and RAD52 or RAD3 gene, or between the bacteriophage λ pL promotor and the RAD52 and RAD3 genes. The fused genes will be introduced into either yeast or E. coli and expressed to overproduce the gene product. The crucial feature of this approach is that expression of the gene will be well controlled; therefore, the gene product will be detected even if it rapidly turns over in vivo.

One radiation-sensitive mutant of yeast, rad18-1, has high spontaneous mutability (mutator phenotype). It is possible that the RAD18 gene product is one of the accessory proteins of yeast DNA polymerase I (a subunit of the DNA polymerase I holoenzyme). Thus, DNA polymerase I holoenzymes will be purified from both wild-type and rad18 cells to compare their subunit structures and accuracy of the DNA polymerization reactions. In the meantime, the RAD18 gene will be cloned.

Recent Accomplishments and Significance for Biomedical Research: By a slight modification of our in vitro DNA replication system, we have developed an in vitro recombination system using yeast delta sequences from yeast crude extract. This system has been extensively characterized. It requires ATP, magnesium chloride and supercoiled plasmid DNA (which consists of at least two delta sequences), and the main product of the reaction was a small circular DNA. The reaction depends on the RAD52 and RAD3 products. Therefore, this system should make possible the identification and purification of these two gene products.

Three different single-stranded DNA binding proteins (14,000, 20,000 and 38,000 daltons) have been purified to homogeneity and polyclonal antibodies have been raised against each. To test whether the purified DNA-binding proteins are required for recombination and/or repair, the genes have been identified and cloned from a yeast genomic library in a λ gt11 expression vector using the antibodies. Then, deletions of the genes have been constructed and integrated into the yeast chromosome and the wild-type genes have been replaced by the mutagenized genes. Although each gene has been disrupted, no phenotype changes have been detected. Therefore, mutants carrying simultaneous deletions of two and three of these genes are under construction.

It is well known that the RAD52 gene product is required for DNA recombination and repair in yeast. Although this important gene has recently been cloned and its nucleotide sequence has been determined, the gene product has not yet been identified and purified. To facilitate the identification and purification of this gene product, we have been pursuing the following approaches. (1) Based on computer analysis of the RAD52 gene sequence, several possible antigenic

oligopeptide regions in the predicted RAD52 protein have been determined. Such oligopeptides have been synthesized chemically, covalently linked to bovine serum albumin and injected into a rabbit to raise antibodies. (2) The second approach was to construct the fusion between the yeast mating type alpha pheromone leader sequence and the RAD52 gene (pMF8-RD52) or between the bacteriophage λ pL promoter and the RAD52 gene (pK333-RAD52). During the expression experiments, we found that the RAD52 gene product is extremely unstable and is rapidly degraded in vivo. This is consistent with the prediction, by computer analysis, of the predicted gene product from the nucleotide sequence.

Plans for Future:

Our in vitro recombination system using yeast delta sequences is very promising. Therefore, we will continue to characterize and fractionate the system to identify and purify various components. Once purified, the components will be characterized and their genes will be identified.

Since we have found that the RAD52 gene product rapidly turns over in both E. coli and yeast, we are going to try to isolate E. coli and yeast mutants which stably maintain the RAD52 gene product. We will also try to clone the RAD18 gene, which may code for one of the subunits of yeast DNA polymerase I.

Publications:

Resnick, M. A., Sugino, A., Nitiss, J., and Chow, T.: DNA polymerases, deoxyribonucleases, and recombination during meiosis in Saccharomyces cerevisiae. Mol. Cell. Biol. 12: 2811-2817, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 61040-01 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Genetic and Biochemical Analysis of Yeast DNA Polymerase I		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: R. K. Hamatake A. Sugino	Staff Fellow Visiting Scientist	LG NIEHS LG NIEHS
Others: A. B. Clark	Biologist	LG NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Genetics		
SECTION Mutagenesis Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 1.25	PROFESSIONAL: 0.75	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>A detailed analysis of the yeast replicative DNA polymerase, DNA pol I, is being undertaken at the molecular and genetic level. The objectives of this project are to map and clone the gene for DNA pol I, to identify subunits and accessory proteins that influence DNA pol I activity and, ultimately, to identify the proteins that regulate its activity on native DNA templates and to determine the mechanism of their interactions.</p> <p>Using purified DNA pol I, we have identified several proteins that stimulate its synthetic activity. These include three different RNase H proteins, three different single-stranded DNA binding proteins and a DNA-dependent ATPase (ATPase III) that possesses a helicase activity.</p> <p>An aphidicolin-sensitive (aph^S) strain of yeast has been used to identify several possible DNA pol I clones. Acquisition of the pol I gene in a high copy number plasmid would presumably confer an aphidicolin-resistant phenotype to the transformed aph^S cell. Analysis of aphidicolin-resistant transformants showed that three different DNA sequences transformed cells to aphidicolin resistant. All three produced slightly increased levels of pol I activity in crude extracts. These DNA sequences are being subcloned to determine the ends of the aphidicolin-resistance genes by deletion mapping. An internal fragment of the aphidicolin-resistance gene will then be cloned into the yeast integration vector YIp5 for a gene disruption experiment to determine the requirement of the intact gene for cell viability. The pol I gene is expected to be essential for viability.</p>		

Research Project:

Nature of Problem: Yeast DNA polymerase I (Pol I) is the replicative DNA polymerase. Because DNA synthesis occurs only in the S-phase of the cell-division cycle, Pol I activity must be controlled. In procaryotes, there are many protein factors that influence and regulate the activity of their replicative DNA polymerases. In yeast, such control is also expected to involve many different proteins. These proteins, the nature of their interactions with yeast Pol I and how they regulate Pol I activity on native templates are the subjects of this research project.

Objectives: The objective is to elucidate the elements affecting and regulating yeast Pol I and to determine their mechanisms of actions.

Experimental Approach and Scientific Justification: Purified yeast Pol I is being used to identify proteins that stimulate its synthetic activity on various defined DNA templates such as activated calf thymus DNA or primed ssDNA. Stimulation of synthetic activity was chosen because it is easily measured and it is known that accessory proteins of procaryotic DNA polymerases have stimulatory effects on synthesis in vitro.

The extensively purified yeast Pol I will be used to detect and isolate proteins that stimulate synthesis on defined templates. This approach has been useful in identifying a yeast DNA primase required for Pol I activity when unprimed ssDNA is the template. In vivo, however, ssDNA resulting from replication fork movement is likely to be coated with single-stranded DNA binding proteins. Therefore, the effect of yeast single-stranded DNA binding proteins on the reaction catalyzed by DNA primase-Pol I on ssDNA will be explored. Templates resembling a replication fork will be constructed and used to probe the requirements for simultaneous strand separation and strand elongation. Such a replication fork template may be obtained by annealing the (-) strand of bacteriophage M13mp18 RF DNA (linearized with a restriction enzyme) to circular (+) strand M13mp19 DNA. These two DNAs are complementary except for the 54-base multiple cloning sites which are in opposite orientations. The annealed products will therefore have a single-stranded gap on the (+) strand and a single-stranded tail from the (-) strand.

Large amounts of purified Pol I enzyme are required to detect and characterize the proteins that stimulate Pol I activity. The ever-present problem of proteolysis in yeast and of low yields has made purifying Pol I very laborious. For this and other reasons, we are attempting to clone the gene for yeast DNA Pol I in order to overproduce it in and purify it from E. coli. The method of cloning utilizes the sensitivity of Pol I to inhibition by the drug aphidicolin. A yeast mutant permeable to aphidicolin is unable to grow in the presence of the drug. Acquisition of the gene for Pol I in a high copy number plasmid may have a gene dosage effect that will allow the cells to grow in the presence of aphidicolin. Thus, aphidicolin-resistant transformants will be isolated from a yeast genomic library and the insert DNA conferring the aphidicolin resistance will be characterized. At the same time, a yeast strain possessing a Pol I enzyme mutated to aphidicolin-resistance is required to confirm that any

aphidicolin-resistance genes code for Pol I. Transformation of the aphidicolin-resistant mutant with a fragment of the Pol I gene in the integrating vector YIp5 should result in the replacement of the aphidicolin-resistant Pol I gene with a wild-type Pol I gene. The resulting transformant would then be aphidicolin-sensitive and would contain an aphidicolin-sensitive Pol I activity.

Recent Accomplishments and Significance to Biomedical Research: Yeast Pol I has been extensively purified, used for raising antibodies and antibody-linked Sepharose has been made to purify a large amount of Pol I enzyme in a short time and with minimum effort. At the same time, several proteins which stimulate the purified Pol I have been purified. These include three different single-stranded DNA binding proteins (14,000, 20,000 and 38,000 daltons), RNase H (68,000 daltons) and DNA-dependent ATPase (63,000 daltons). The nature of these stimulation reactions has been investigated. This study strongly suggests that RNase H and DNA-dependent ATPase are subunits or accessory proteins of Pol I.

Transformation of the aphidicolin-sensitive permeable mutant with a yeast genomic DNA library in a high copy number shuttle vector resulted in several different transformants resistant to aphidicolin. Four plasmids, two of which share common DNA sequences, contain the aphidicolin-resistant DNA sequences. Deletion mapping to determine the ends of the aphidicolin-resistance genes will be followed by gene disruption experiments to determine if these sequences are required for cell viability. Any aphidicolin-resistance gene that is not essential is very unlikely to be the gene for DNA Pol I.

Plans for Future:

Enzymology of DNA Synthesis Involving DNA Pol I: (1) We will continue to identify protein factors that stimulate pol I synthetic activity. Previously overlooked column fractions (such as ssDNA-cellulose flow-through fractions) may contain stimulatory factors. More extensive purification of pol I fractions may also reveal more proteins that interact with pol I. (2) Templates and reaction conditions that approximate isolated steps during DNA synthesis will be developed. Primed and unprimed ssDNA will serve as templates for leading and lagging strand DNA synthesis. The addition of ssDNA binding proteins and other pol I accessory proteins may more faithfully mimic in vivo conditions. The enzymology of DNA synthesis at a replication fork will be studied using a double-stranded circular template with a single-stranded gap and a single-stranded tail. Pol I, after filling in the gap, will encounter conditions similar to a replication fork. The protein factors required for strand separation may then be determined using this template.

Cloning and Genetics: (1) Once isolated, the mutant strain containing an aphidicolin-resistant DNA pol I will be mapped to determine the locus for the pol I gene. (2) After verifying that the pol I gene has been cloned, we will attempt to have it expressed and over-produced in *E. coli*. (3) The cloned pol I gene will also be used for site-directed in vitro mutagenesis in order to isolate temperature-sensitive pol I mutants. These ts mutants will be used to isolate extragenic suppressors to detect and study proteins that interact with pol I in vivo.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 65021-13 LG																		
PERIOD COVERED October 1, 1984 through September 30, 1985																				
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Investigation of Germinal Mutation Induction in Mice																				
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: F. M. Johnson</td> <td style="width: 33%;">Research Geneticist</td> <td style="width: 33%;">LG, NIEHS</td> </tr> <tr> <td>Others: L. C. Skow</td> <td>Senior Staff Fellow</td> <td>LG, NIEHS</td> </tr> <tr> <td>M. L. Snell</td> <td>Bio. Lab. Technician</td> <td>LG, NIEHS</td> </tr> <tr> <td>Marjo Smith</td> <td>Postdoctoral Fellow</td> <td>LG, NIEHS</td> </tr> <tr> <td>D. P. Lovell</td> <td>Statistician</td> <td>BIBRA</td> </tr> <tr> <td>S. E. Lewis</td> <td>Senior Geneticist</td> <td>RTI</td> </tr> </table>			PI: F. M. Johnson	Research Geneticist	LG, NIEHS	Others: L. C. Skow	Senior Staff Fellow	LG, NIEHS	M. L. Snell	Bio. Lab. Technician	LG, NIEHS	Marjo Smith	Postdoctoral Fellow	LG, NIEHS	D. P. Lovell	Statistician	BIBRA	S. E. Lewis	Senior Geneticist	RTI
PI: F. M. Johnson	Research Geneticist	LG, NIEHS																		
Others: L. C. Skow	Senior Staff Fellow	LG, NIEHS																		
M. L. Snell	Bio. Lab. Technician	LG, NIEHS																		
Marjo Smith	Postdoctoral Fellow	LG, NIEHS																		
D. P. Lovell	Statistician	BIBRA																		
S. E. Lewis	Senior Geneticist	RTI																		
COOPERATING UNITS (if any) Research Triangle Institute, Life Sciences Group, Research Triangle Park, N.C.; British Industrial Biological Research Association, Carshalton, Surrey, England																				
LAB/BRANCH Laboratory of Genetics																				
SECTION Eukaryotic Gene Structure Section																				
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																				
TOTAL MAN-YEARS: 5.0	PROFESSIONAL: 3.0	OTHER: 2.0																		
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews											
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The objective of this project is to detect natural and induced mutations in mice for the purpose of providing understanding of the specific molecular events involved in germinal mutation and the effects of these events on the life, form and function of the mammalian organism. Results are relevant to cases of human exposures to mutagens and the potential for increased risk of genetic disease that may accompany mutagen exposure. The problem is approached by detecting mutations at specific biochemical loci with electrophoretic methods, by conducting characterization studies on the mutant genes and gene products, and by examining the animals for expressed physical abnormalities correlated with mutation rate increases and with specific induced-mutant genotypes. The methods have led to successful identification of more than 20 ethylnitrosourea-induced mutants affecting proteins such as malic enzyme, α hemoglobin and phosphoglucosmutase, but there is little evidence to suggest that the induction of the detected mutations has been accompanied by any increased incidence of adverse gene expression. Perhaps the best example of a significant genetic disorder (with homology in man) we have discovered by electrophoresis (a β-thalassemia) was found to have originated spontaneously. Furthermore, our most recent analysis of skeletal variation shows a reduction in the frequency of naturally occurring variation as the statistically most significant effect of mutagen treatment. The results raise questions as to the extent hypotheses of human genetic risk based upon increased mutation rates are indicative of elevated probabilities for significant genetic damage. </p>																				

PROJECT DESCRIPTION

NATURE OF THE PROBLEM: Current perceptions of human genetic risk implicate elevated mutation rates as a source of increased genetic disease in human populations subjected to exposure to mutagenic agents in the environment. Although many test methods provide for the efficient detection of mutations, the mouse affords unique experimental opportunities to analyze efficiently the effects of mutations in a mammal with many established homologies with man. Therefore with mice, an effective means to detect mutations and to provide understanding of their effects in terms relatable with human gene expression is available.

APPROACH AND OBJECTIVE: Our work has concentrated upon detecting induced and spontaneous mutations in mice using electrophoretic techniques applied to surgically removed tissue samples. After mutant identification, efforts to characterize the variation are undertaken. Characterization includes inheritance studies, analyses of structural alterations in the DNA and gene products, comparative functional analyses of the mutant and normal gene product, and investigations as to how the alterations may affect the life of the animal. The progeny of both mutagen-treated and control parental animals are used. The principal mutagen we have used is ethylnitrosourea.

Subsets of animals derived from the electrophoretic studies have been used to analyze morphological variation in the skeleton. By comparing control and mutagen treated groups it is possible to determine the extent to which increased variation and increased severity of expression is associated with mutagen exposure. Quantitative measurements and multivariate statistical techniques are used. Having mutation frequencies obtained from the electrophoretic studies, any increased morphologically expressed variation can then be correlated with average induced-mutation rates. Furthermore, if correlation is established, corroboration of induced detrimental effects is available from the results of analyzing the effects of the mutations at specific biochemical loci and from genetic studies of the morphological variation.

JUSTIFICATION: According to published approaches to human genetic risk estimation it has been suggested that variously obtained induced mutation-rate data and certain dominant indicators of genetic damage in mice may provide a suitable basis for achieving useful numerical risk values. Once correlation is established, it has been further suggested that short-term mutagenesis tests may alone be sufficient to estimate genetic risk, obviating the need for more time consuming and expensive investigations with mice. Human exposures to agents determined to have the potential for causing increased risk of genetic damage may need to be controlled by law. Our project with mice provides a test of the principle assumption on which classical population genetic (load) theory rests and on which the validity of testing strategies for predicting human mutagenic risk depend. Proper validation is important because particular mutagen exposures (to X-rays for example) have well established therapeutic aspects. Regulations based upon invalid risk estimates would, thus, introduce potential health hazards to those individuals who would be deprived of beneficial mutagen exposures. Our project secondarily bears on carcinogenic

risk assessment. A variety of Federal and State laws that regulate human exposure to toxic substances are justified, in part, on observed correlations between mutagenicity and carcinogenicity, the practical interpretation of which is highly uncertain.

RECENT ACCOMPLISHMENTS: A large series of electrophoretically recognized ethylnitrosourea-induced mutants have been recovered from the experiments. Several spontaneous mutations have also been identified. The average mutation rate caused by a single 250 mg/kg dose of ethylnitrosourea has been determined to be 100 times, or more, greater than the spontaneous mutation rate. The evidence for a significant detrimental effect accompanying the mutation rate increase has, however, not been compelling. Most of the induced specific-locus mutations do not show demonstrable deleterious effects even though the majority of the mutants appear to be different from previously recognized natural genetic variants. Additionally, morphologically (skeletal) variation is not significantly increased by mutagen exposure. A recently completed and much larger follow up study to our original investigation actually shows an appreciable and highly statistically significant reduction in morphological variation among the offspring of ENU-treated male parents. These findings suggest highly elevated mutation rates may sometimes occur in the absence of important adverse genetic expression. More mouse data are needed. If future work confirms present findings, a practical implication of the results would be that meaningful alternative approaches to currently used genotoxicity methods, as applied widely to chemicals, complex environmental mixtures and the like, would need to be sought in order to properly protect public environmental health. We have recently completed a small mutagenesis study, involving ethylene oxide and found some dominant physical variants induced in post meiotic male germ cells. A few induced electrophoretic mutants were also recovered. Comprehensive morphological analysis was not done on this material, so the experiment is uninformative as to whether the detected morphological variation represents a true incremental increase in the overall range of normally expressed morphological variation. Post-meiotic exposures to high doses of various agents are known to cause heritable chromosome alterations, and associated early embryonic dominant lethality, infertility and dominant morphological abnormality. However, the extent to which such risk exists for exposures to any agent at doses below which a general toxic or reproductive effect can be readily differentiated remains to be determined and a central issue from both the theoretical and practical sides of the genetic risk problem.

PLANS FOR FUTURE: We are beginning an experiment with X-rays; the length of this experiment will probably exceed 5 years. Analysis will follow along the lines of previous work.

Previously published mammalian-genetic, carcinogenesis and short-term test, and epidemiological data will be reexamined to determine if new interpretations are possible, and laboratory experiments involving the use of other mutagens and more powerful detection techniques will be planned.

PUBLICATIONS

Lovell, D.P., and Johnson, F.M.: Quantitative genetic variation in the skeleton of the mouse. I. Variation between inbred strain. Genetical Research 42: 169-182, 1984.

Lovell, D.P., Totman, P., and Johnson, F.M.: Variation in the shape of the mouse mandible. I. Effect of age and sex on the results obtained from the discriminant functions used for genetic monitoring. Genetical Research, 43:65-73, 1984.

Lovell, D.P., Willis, D.B., and Johnson, F.M. Lack of evidence for skeletal abnormalities in offspring of mice exposed to ethylnitrosourea. Proc. Natl. Acad. Sci. 82:2852-2856, 1985.

Popp, R.A., Popp, D.M., Johnson, F.M., Skow, L.C., and Lewis, S.E. Hematology of a murine beta thalassemia. A longitudinal study. Ann. NY Acad. Sci. (In Press).

Lewis, S.E., Johnson, F.M., Skow, L.C., Popp, D.M., Barnett, L.B., and Popp, R.A. A mutation in the β -globin gene detected in the progeny of a female mouse treated with ethylnitrosourea. Proc. Natl. Acad. Sci. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 65033-02 LG									
PERIOD COVERED October 1, 1984 to September 30, 1985											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) In Vivo Mammalian Mutagenesis											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">H. V. Malling</td> <td style="width: 35%;">Research Geneticist</td> <td style="width: 15%;">LG, NIEHS</td> </tr> <tr> <td></td> <td>J. G. Burkhardt</td> <td>Research Chemist</td> <td>LG, NIEHS</td> </tr> </table>			PI:	H. V. Malling	Research Geneticist	LG, NIEHS		J. G. Burkhardt	Research Chemist	LG, NIEHS	
PI:	H. V. Malling	Research Geneticist	LG, NIEHS								
	J. G. Burkhardt	Research Chemist	LG, NIEHS								
COOPERATING UNITS (if any) <table style="width: 100%; border: none;"> <tr> <td style="width: 60%;">C.A. Hutchinson III, M.H. Edgell, & S.C. Hardies UNC at Chapel Hill Chapel Hill, N.C.</td> <td style="width: 40%;">E. J. Eisen North Carolina State University Raleigh, N.C.</td> </tr> </table>			C.A. Hutchinson III, M.H. Edgell, & S.C. Hardies UNC at Chapel Hill Chapel Hill, N.C.	E. J. Eisen North Carolina State University Raleigh, N.C.							
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LAB/BRANCH Laboratory of Genetics											
SECTION Eukaryotic Gene Structure											
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709											
TOTAL MAN-YEARS: 2.3	PROFESSIONAL: 2	OTHER: 0.3									
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> This project is to study gene mutations and small intrachromosomal changes directly in exposed mammals. We have taken two approaches. 1). Site specific alterations in proteins detected in single cells: Antigenic differences between rat and mouse LDH-C were used to detect mutations in mouse sperm. Initial work indicated that this system had potential for the study of mutagenesis in mammals; later research showed that the apparent mutation frequency varied too much to give reproducibly useful data. The conclusion was reached that although antibodies had the needed sensitivity, interspecies differences may not be appropriate as markers for induced mutations; also, a model mutant cell must exist for a proper test of antibodies. Therefore, induced mouse mutants with changes in electrophoretic mobility of enzymes were selected for these studies. such mutants exist in both MOD-1 and LDH-A and both enzymes are present in sperm. The LDH-A mutants are presently being investigated. 2). Site specific changes detected at the DNA level: A major problem for detection of genetic damage directly in mammalian DNA is that most genes occur in one, or few copies; there are however, two possible approaches. The first is the use of mitochondrial (mt) DNA. Cloned mouse mtDNA has been used for restriction analysis of sperm mtDNA isolated from a single mouse; after additional technical improvements, the mtDNA from treated mice will be examined for mutations. The second is the use of viral DNA transformed into mammalian DNA. Double stranded DNA from ϕX174 am3, cs70 was transformed into mouse L-cells. The DNA was incorporated into several places in tandem arrangements. Using restriction enzymes and ligase it was possible to transfect spheroplasts with ϕXDNA from the transformed mammalian cells. Attempts are being made to create a mouse strain with ϕXDNA in the genome for the study of mutation induction in any part of the animal tissue. </p>											

PROJECT DESCRIPTION

NATURE OF THE PROBLEM: The primary problems to be investigated are the mechanisms of mutations, the differential sensitivity of various loci to mutagenesis, the relationship of mutations to the organization of the genome, and the phenotypical consequences of mutations. Mutations may arise from a series of different mechanisms such as a direct chemical change in the DNA caused by reaction of the agent with the DNA, its precursors or the enzymes involved in DNA replication or repair, or through more genetically dependent mechanisms including gene conversions or movement of transposable elements. The relative roles that these mutational processes play in the production of spontaneous and induced mutations is not known. In addition, these events occur within a context of the physiological and metabolic processes of the mammal; a clear understanding and evaluation of somatic or germinal mutations requires that a proportion of our study be directed at in vivo mammalian mutagenesis.

Given the mutation frequencies and the number of useful markers, whole animal approaches would seem to have only marginal feasibility. However, data from microbial systems suggest the possibility that given an induced frequency and the number of mammalian genes, every offspring in an exposed population could be the carrier of a new mutation. Yet, our capacity to detect and understand these events in mammals is limited. Development of systems for detection of mutations in cells from the exposed individual (animal or human) would be extremely important for understanding the genetic damage caused by exposure to mutagenic agents and for elucidating mutation mechanisms in the mammal. Efforts are in two basic categories, 1) site specific alterations of proteins with detection in single cells and 2) site specific changes detectable directly at the DNA level.

OBJECTIVES OF RESEARCH PROJECT IN NEAR TERMS: There are plenty of methods for study of chromosome aberrations in single cells in vivo, but very few methods exist for obtaining point mutations or small intrachromosomal changes from single cells from laboratory mammals or humans. Study of the relationship of mutations to the organization of the genome may require isolation of many different types of mutations within at least a few genes. Using single cells obtained from the exposed individual may be the best approach to obtain a broad spectrum of mutations. Different genetic markers are being explored and evaluated for their suitability in these studies.

EXPERIMENTAL APPROACHES AND SCIENTIFIC JUSTIFICATION: The following approaches are taken. a) Use of altered proteins as markers for genetic changes in single cells. Selection of markers for study of mutagenesis in single cells in the exposed individual is an important step. The past experience in this area has provided valuable information for guiding the selection. At present, the MOD-1 and LDH-A loci seem promising candidates in mice. Use of these markers has the following advantages. (a) Transmissible mutations induced by ENU have been isolated. (b) The enzyme is present both in germinal and somatic cells. (c) Electrophoretic differences exist between various mouse strains for MOD-1, and (d) the mutants isolated after ENU treatment are likely to be due to single base-pair changes. An important feature is that heterozygotic animals carrying a wild-type allele and a mutant allele can be produced. Cells from the

heterozygote should simulate the mutant cells in the exposed animal. The antibodies, therefore, can be adjusted to detecting these cells, and the entire approach thoroughly tested. This was one of the essential features lacking in the LDH-C system. If antibodies can be isolated that are specific to the mutants, it should be possible to detect mutation both in somatic cells and sperm. This would enable a comparison of somatic, germinal, and transmissible mutation rates. Molecular analysis of the transmissible mutants will be attempted. The search will continue for other markers. Any individual marker that is selected may possess some unforeseen technical difficulties.

Therefore, it is important that several markers are simultaneously developed. b) Use of viral transformed cells for study of site specific mutations in mammals. Mouse L-cells were transformed with double stranded RF forms of ϕ X174 am3 cs70. Both markers can be used for study of reverse mutations. The phage DNA can be isolated from the mammalian DNA and be used for transfection of spheroplasts. It should be possible to study mutation induction in the viral DNA that is incorporated into the mammalian DNA. This DNA is exposed to the same metabolic activation enzymes and repair enzymes as the normal mammalian DNA. Although the viral DNA lacks some features of mammalian DNA such as repetitive DNA, it nevertheless should be possible to obtain accurate mutation information at specific sites. c) Use of mitochondrial DNA for study of genetic damage. The mitochondrial DNA (mt-DNA) in mammalian organisms has features that are different from the nuclear DNA. The mt-DNA is circular, evolves faster, is less repaired, and usually shows maternal inheritance. Genetic diseases are known in humans that are traceable to mutations in the mitochondrial DNA. Analysis of mitochondrial DNA from sperm of mutagen treated and untreated male mice is one of the approaches taken. We are exploring the possibility that the lack of α -GPD activity is due to changes in the mitochondrial DNA similar to those found in petite mutants in yeast.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: a) Markers for single cell *in vivo* mutagenesis: The normal MOD-1 enzyme has been purified and antibodies produced. These antibodies have been used to show that sperm as well as somatic cells will react to the antibodies. This is an important first criteria for the possibility of use as a genetic marker for detection of induced mutations. One form of abnormal MOD-1 has been test purified on a small scale from mice bearing the mutation in homozygous form. In addition, chemically-induced mutants in LDH-A are being obtained from other laboratories and analyzed. b) The viral transformed L-cells: Preparation of spheroplasts are laborious and time consuming and the competence of the spheroplasts varies considerably between preparations. In order to circumvent this problem, a technique was developed to maintain their competency for at least nine months. DNA from mouse L-cells transformed with ϕ X174 was digested with the restriction endonuclease *pst*-1 and treated with T_4 ligase. This DNA was used for transfection of spheroplasts and ϕ X174 plaques were recovered from this DNA. We will develop techniques for isolating the ϕ X174 DNA from mammalian cells and in the future treat the L-cells transformed with ϕ 174 am3 cs70 with various mutagens. Reverse mutations of the am3 and the cs70 will be measured among the phages recovered from DNA from treated and untreated cell cultures. c) Mitochondrial DNA: The selection of mt-1 DNA from mouse sperm for study of genetic damage in mammals is related to the following observation: α -glycerophosphate dehydrogenase (α GPD) is a nuclear-coded enzyme located between the inner and outer membrane of

mitochondria. A high frequency of sperm from mice treated with certain mutagens lack α -GPD activity. The frequency varies with the treated stage of spermatogenesis. The highest frequency coincides with the replication of DNA in the spermatids. One possibility is the lack of α GPD activity may be related to damage to the mitochondrial DNA. Mitochondrial DNA has been isolated from sperm taken from the vas deferens of a single mouse (total approximately 10^7 sperm). Restriction patterns in this DNA have been visualized in mt DNA from approximately 10^5 sperm. Cloned mt-DNA has been accumulated. The sensitivity of the system may enable us to detect changes in the mt-DNA after mutagenic treatment seen as changes in restriction enzyme patterns. If this system is successful it should open up the possibility for study of damage of the mt-DNA in mammals in vivo.

PLANS FOR SUBSEQUENT YEARS: a) The MOD-1, LDH-A and other markers for single cell in vivo mutagenesis: The genetic damage in the mutants will be studied at the molecular level, the results will be compared to both spontaneous mutations and induced mutations in other markers. Attempts will be made to obtain heterologous antibodies to both wild type and mutant proteins starting with induced mutations of MOD-1 and LDH-A and natural variants of MOD-1. In collaboration with Dr. Lohman (Netherlands) we hope to obtain monoclonal antibodies. Attempts will be made to adapt the system for the cell sorter. The enzymes are present in both sperm and somatic cells. If the approach is successful then data can be obtained for mutation frequencies in various tissues; it will also be possible to compare transmissible mutation rates, and single cell mutation rates in somatic and germinal tissue. These data may assist evaluation of mutation monitoring data from the human population. b) The viral transformed L-cells: Attempts will be made to concentrate the viral DNA (ϕ X174) from the mouse L-cells. If this is successful, ϕ X174 will be recovered from treated and untreated cells. In collaboration with the scientists at UNC, attempts are being made to transfect fertilized mouse eggs with ϕ X174, which hopefully will result in mice with ϕ X incorporated into their genome. Attempts will also be made to utilize other viral vectors such as lambda phage. This project may result in a system where site specific mutagenesis can be measured in any organ of a mouse from which DNA can be obtained in sufficient quantities. If successful, it may also enable use to understand mutation fixation in mammalian genome. It may also give very precise data of the sensitivity of the various sperm stages to mutagenic treatment. Knowledge of parameters about mutagenesis in a mammal is likely to give information about the damage induced by exposing the mammalian organism to a mutagen. c) The mt-DNA: If the hypothesis that the lack of α -GPD activity in mutagen treated animals indicates damage to the mt-DNA, then it should be possible to evaluate the potential effect of mutagens on mt-DNA. The DNA will be cloned and restriction mapping (tentatively) used to identify the damage at the molecular level. Attempts will also be made to compare the profile of the proteins from treated and untreated animals. The sperm from the F_1 generation originated from either treated males or female will be studied for damage in the mt-DNA.

PUBLICATIONS

Burkhart, J.G., Benziger, J., Svenson, K. and Malling, H.V. An evaluation of heterologous antibodies to lactate dehydrogenase-C in the detection of mutations. Mut. Res. 148:135-149, 1985.

Burkhart, J.G. and Malling, H.V.: Differentiation of Binuclear ("Diploid") Spermatozoa. Submitted.

Saxton; A.L., Eisen, E.J., Johnson, B.A. and Burkhart, J.G. A new mutation causing hereditary jaundice in mice. J. of Heredity (In Press), 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 65034-01 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Specificity of Spontaneous and Induced Mutation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. M. Schaaper

Visiting Associate

LG NIEHS

R. L. Dunn

Biologist

LG NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park North Carolina 27709

TOTAL MAN-YEARS:

1.25

PROFESSIONAL:

0.25

OTHER:

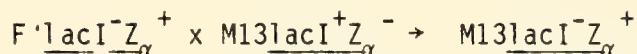
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☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The specificity of mutation is being studied by recording the spectrum of mutational changes in large groups of E. coli lacI⁻ mutants obtained by DNA sequence analysis. Sequence analysis is facilitated by the recovery of the mutant lacI genes by genetic recombination from F'lac onto an M13 vector which carries both the lacI gene and a defective lacZ_α gene (M13 lacI⁺Z_α⁻):



This recombinant phage can be easily distinguished from its parent by its ability to perform α-complementation in the absence of the inducer IPTG. This new procedure has been applied to the production of a spectrum of spontaneous lacI mutations to serve as a baseline for future specificity studies. The spontaneous spectrum is characterized by the occurrence of at least six different classes of mutations. These include (frameshift) hotspot mutations, deletions, base substitutions, IS1 insertions, single-base frameshifts, and duplications. The relative frequencies of the non-hotspot events in lacI are taken to be representative of their general importance in spontaneous mutagenesis.

Research Project:

Nature of the Problem: The mechanisms by which cells produce mutations appear varied and complex. Mutations are expected to occur spontaneously as a consequence of incorporation errors during DNA replication or DNA repair, or via independent pathways such as DNA rearrangements or insertion elements. Spontaneous DNA damage is also expected to play an important role, either by causing direct mispairing or by eliciting DNA repair which in itself can be mutagenic. In the case of induced mutagenesis this situation is even more complicated due to the wide spectrum of damages generally produced in DNA. Each individual type of DNA damage has its own target distribution, miscoding vs noncoding properties, susceptibility to removal by DNA repair, tendency to induce error-prone SOS-repair, and capacity to be a promutagenic lesion during SOS-repair. For a full understanding of the mutational mechanisms, information on the specificity of the mutational process is a prerequisite. This project is directed at providing exact DNA sequence information on spontaneous and induced mutations in the bacterium E. coli.

Objectives of Research Project: The objectives of this project are to collect large numbers of mutants in the lacI gene of E. coli and to determine at the DNA level the sequence changes that they represent. The mutational spectrum thus obtained defines the specificity of the mutagenic process and can be analyzed to reveal clues regarding the underlying mechanisms.

Experimental Approach and Scientific Justification: In this project E. coli lacI mutant are selected as cells that express the lac operon constitutively (P-gal selection). To obtain DNA sequence information on these mutants a procedure is applied which was specifically developed as part of this project. In this procedure the lacI gene residing on F'lac is, by genetic recombination, recovered on a M13lac derivative. On this vector the mutant gene is immediately accessible for DNA sequence analysis by the dideoxy chain termination method.

Recent Accomplishments and Significance to Biomedical Research: A rapid recovery system for episomal lacI mutations was developed by construction, via recombinant DNA techniques, of mRS81, an M13 derivative which carries the entire lacI gene as well as the α -complementation region of the lacZ gene. In addition, a single point mutation was introduced in the lacZ _{α} gene abolishing its α -complementing ability (M13lacI⁺Z _{α} ⁻). The recovery scheme is based on the following formula: $F' \text{lacI}^- \text{Z}_{\alpha}^+ \times \text{M13lacI}^+ \text{Z}_{\alpha}^- \rightarrow \text{M13lacI}^- \text{Z}_{\alpha}^+$. The resulting recombinant phage can be easily distinguished from its parent by its ability to perform α -complementation in the absence of the inducer IPTG. The procedure is rapid and has a low background frequency due to the requirement for a double change in order to express the selected phenotype. This recovery scheme has been applied to a collection of 176 spontaneous mutants. The results showed the diversity of spontaneous events, six different classes being discovered. Two-thirds of all lacI mutations occurred at the lacI hotspot site containing a tandem triple repeat of four bases. Our analysis of the local DNA sequence suggests that the origin of these events may depend on structural features beyond those permitted by the repeated tetramer. The non-hotspot mutations comprised deletions (39%), base substitutions (35%), IS1 insertions (12%), single-base frameshifts (9%) and duplication mutations (5%). Considering the

size of the target gene (1100 bp), these values are likely to be representative for spontaneous mutation in general. The results obtained for individual classes will be valuable for the analysis of the origin of these mutations. In addition, the results form a baseline against which to compare spectra of induced mutagenesis.

Plans for Future:

The recovery method for $lacI^-$ mutations as described above allows the rapid accumulation of detailed information on the specificity of mutational processes, and is therefore an exquisite tool to study the mechanism of mutation. We intend to study the mechanisms of UV-induced mutation by recording mutational spectra. These spectra are expected to be helpful for determining the nature of the premutagenic lesion after UV-irradiation, i.e. the cyclobutane pyrimidine dimer or the more recently discovered pyrimidine-pyrimidine (6-4) photoproduct. In addition, the inclusion of an excision-repair-deficient strain will provide information on the role of this mode of repair in UV mutagenesis.

A second study that we plan to perform is the recording of $lacI$ mutational spectra in *E. coli* muth, mutL and mutS strains. These strains each have a deficiency in the process of post-replicative methylation-instructed mismatch repair, and consequently are strong mutators. These spectra should not only yield important information on the specificity of the correction process, but should also reveal the spectrum of uncorrected DNA replication errors.

Publications:

Schaaper, R.M., Danforth, B.N. and Glickman, B.W.: Rapid repeated cloning of mutant lac repressor genes. Gene, 1985 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 65035-01 LG
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Specificity of Mutagenesis in Mammalian Genes Using a Natural Gene		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: P. J. de Jong Visiting Fellow LG NIEHS		
COOPERATING UNITS (if any) Dr. B. W. Glickman Biology Department, York University Downsview, Ontario, Canada		
LAB/BRANCH Laboratory of Genetics		
SECTION Mutagenesis Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Environmental mutagens are able to induce mutations in the genomes of pro-karyotes and eukaryotes in a non-random fashion with regard to the DNA sequence. An understanding of this non-random character of mutagenesis will eventually lead to better estimates of the risks presented by environmental mutagens. To study the mutational specificity in mammalian cells at the molecular level, the <u>adeninephosphoribosyltransferase (aprt)</u> gene from Chinese Hamster Ovary cells was chosen as a model system. This gene has been cloned previously. Specific advantages of this system include the relatively small gene size (completely contained in a 3.8 kb BamHI restriction fragment) and the well characterized selective growth procedures for <u>aprt</u>-deficient cells. In addition, a hemizygous cell-line (D422) is available which has a complete deletion of one of the two <u>aprt</u> alleles. Using this cell line, a direct correlation can be made between the mutant phenotype and the remaining <u>aprt</u> allele. About 100 spontaneous and 60 X-ray-induced mutants have been selected so far. To permit rapid cloning of the mutant alleles, several new cloning vectors have been constructed and methods for repetitive mammalian gene isolation have been optimized. In a preliminary test of the procedures, 30 <u>aprt</u> alleles were isolated, which represent 21 independent spontaneous mutants. Several of these mutants have been analyzed by rapid DNA sequencing techniques.</p>		

Research Project

Nature of the Problem: To gain a better understanding of the risks presented by environmental mutagens, precise molecular analysis of the mutagenic process will be required. This analysis should include information on premutational lesions, cellular repair capacities and the nature of the eventual mutation. Previous attempts have mainly been focussed on mutagenesis in bacterial systems and have resulted in an impressive database concerning the DNA sequence specificity of mutagenic processes. As yet, very little is known about mutagenic specificity in mammals, which are more relevant for risk analysis. This lack of information is mainly due to the technical problems associated with the molecular analysis of the complex mammalian genome. Recent advances in DNA technology, however, permit a more extensive investigation.

Objective: The aim of this project is to develop procedures to determine the DNA sequence alterations occurring in response to a mutational treatment. This requires the construction of genomic libraries, the isolation of the mutant gene from the library, and subsequent DNA sequence analysis. An appropriate target gene has to be chosen, one which provides the cell with a distinct, easily selectable mutant phenotype. It is also preferred that only one active gene copy be present and that pseudogenes be absent. (Pseudogenes complicate gene isolation from recombinant DNA libraries.) The aprt gene in CHO cells is uniquely suitable for these purposes. It has a small size (less than 3.8 kb) and the wild-type gene has already been cloned. Using the cloned gene as a probe for hybridization experiments, no pseudogenes could be detected. A hemizygous cell line is available (line D422), which has a complete deletion of one of the aprt alleles. In addition, the gene is non-essential under normal growth conditions because it is involved in only one of the two pathways for adenine nucleotide synthesis. Therefore, a wide variety of gene-inactivating mutations might be obtained without affecting the viability of the cells. Mutants can be selected with high plating efficiencies under conditions which are highly toxic for wild-type cells.

Approach: Tissue culture cells with a complete deficiency of adeninephosphoribosyltransferase activity can be grown selectively using media supplemented with 8-azaadenine (8-AA), an adenine analog which upon phosphorylation becomes toxic to the cells. $APRT^+$ cells rapidly lose viability in the presence of 8-AA, whereas $APRT^-$ cells are not affected as long as cross-feeding of toxic metabolites from $APRT^+$ cells is prevented. For mutagenesis, the hemizygous D422 line was used. In this line, mutants with aprt deficiencies can be isolated at frequencies about two orders of magnitude higher than for the parental diploid cells. For instance, ethylmethanesulfonate (EMS) induces mutations at 2×10^{-6} in diploid CHO cells compared with 4×10^{-4} in the hemizygous D422 cells. To confirm that the mutations occurred in the aprt gene, the ability of the cells to produce adenine nucleotides by the salvage pathway was tested. To this end, the de novo pathway of nucleotide synthesis is blocked with aminopterin and, as a result, the salvage pathway becomes essential. Because cells deficient in $APRT$ activity are also blocked in the salvage pathway, they will not grow in the presence of aminopterin. The mutants we have isolated (100 spontaneous and 60 X-ray induced mutants) fulfill this requirement.

The availability of restriction maps for aprt permits the physical enrichment of gene sequences by size selection of restriction fragments prior to library construction. The wild-type aprt gene is known to be present on a 4.3 kb BglII/HindIII fragment and size-selected fragments of 3-5 kb were therefore used to construct bacteriophage lambda libraries. Consistently large libraries could be prepared using an improved ligation procedure which includes polyethyleneglycol. Bacteriophages with the aprt gene could be obtained from these libraries under selective growth conditions, using an adaptation of the procedures developed by Brian Seed at Harvard. These procedures are based on the use of phage libraries with amber mutations in the essential genes A and B. Lytic infection can only occur in the presence of an amber-suppressor gene in the host or in the bacteriophage genome (e.g., the supF gene). The libraries can, therefore, be propagated on a host containing a small plasmid carrying the supF gene. This plasmid is constructed such that it also includes a "probe-sequence" with homology to sequences flanking the aprt gene on the 4.3 kb BglII/HindIII fragment. During the propagation of the library, aprt-containing lambda phages have the opportunity to get the probe plasmid integrated into the lambda genome by homologous recombination. By the uptake of the supF-containing plasmid, the recombinant lambda is now able to grow on hosts lacking a suppressor gene. Dedicated lambda vectors and probe plasmids have been constructed to allow the use of this recombination-selection scheme. These vectors have been used successfully in a first trial to isolate 30 aprt alleles representing 21 distinct mutants.

For the subsequent DNA sequence analysis of the aprt alleles, further subcloning into a bacteriophage M13 vector is required. (The single-stranded nature of M13 DNA is needed for DNA sequencing by Sanger's protocols.) To facilitate subcloning, a M13 DNA fragment containing the replication origin and packaging signal was included in the probe plasmid and, hence, is also present in the aprt lambda phage with its integrated plasmid. The plasmid sequences, including the M13 fragment and the aprt gene, can be isolated on one restriction fragment by an appropriate restriction enzyme (BamHI). Circularization of this fragment using T4 DNA ligase generates a plasmid which, when present in a suitable bacterial host, can be converted into a single-stranded form by infection of the host with M13. The single-stranded plasmid is packed efficiently into M13 particles. The plasmid-containing particles can be isolated at high yield from the bacterial medium using simple precipitation procedures. DNA sequence analysis of the single-stranded plasmid DNA is possible using a set of short oligonucleotide primers complementary to sites spaced about 200 bp along the aprt gene. All of the alleles isolated in lambda have also been subcloned in plasmids. Several of the mutational alterations in the aprt alleles have been identified.

To further facilitate the subcloning procedures, lambda vectors have been constructed with a direct repeat of the M13 replication origin. Between the repeat sequences, a plasmid has been inserted with a selectable gene and unique restriction sites for library construction. Double infection of host cells with this vector and M13 phage results in the release of the plasmid from the lambda genome in a single-stranded form and its subsequent packaging into M13 particles. High titers of plasmid-containing particles, comparable with M13 viral titers, have been obtained by this double infection protocol. The plasmid particles have been used to establish plasmid-bearing cell lines. The present set

of lambda vectors with M13 repeat sequences does not yet permit the use of the recombination-selection protocol. Further efforts will be directed to the construction of improved vectors. These vectors might also prove useful in the isolation of genes from libraries by direct physical isolation of the corresponding single-stranded plasmid. This will be feasible by hybridizing the single-stranded libraries with biotin-labeled probe sequences. The biotin-labeled partial duplex DNA can then be separated from the bulk of the single-stranded library by binding to streptavidin-agarose resins.

Plans for Future: The work on this project at NIEHS will be terminated before the end of FY 1985 but will be continued and expanded by the cooperating unit in Canada.

Publications:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 65036-01 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Organization and Regulation in *D. melanogaster*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. H. Judd Research Geneticist LG, NIEHS

Others: Patricia S. Davis Chemist LG, NIEHS

Deborah A. Adams P Appointment LG, NIEHS

Katherine M. Peterson Q Appointment LG, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

1.75

PROFESSIONAL:

0.25

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Selected genes in *Drosophila melanogaster* are being studied in an effort to understand their organization and regulation through the analysis of mutations that upset regulatory functions. A locus of particular interest is white, which controls one of the steps in the pigmentation of the eyes, malpighian tubules and testis sheath in *Drosophila*. A large number of mutations, some of which appear to upset the regulation of the locus, have been genetically characterized. The molecular analysis of many of these mutants has also been done, showing that a large proportion of spontaneous changes result from the insertion/deletion of transposable elements. Two aspects of transposon-induced changes at white are being studied. One deals with modifications of regulation brought about by insertion of the transposon BEL. In one class of changes, white function is expressed as a mosaic of normal and mutant pigmented patches in clonal patterns in the eye. Another class exhibits mosaicism but in nonclonal, nonautonomous patterns. Both types of mutations are related by descent, having their origin in the insertion, excision or transposition associated with the BEL transposon. The transposon has been molecularly cloned and mapped by restriction endonucleases and a number of the mutations it caused have been characterized molecularly. Other transposon-induced modifications at white locus are transposon involvements in asymmetrical pairing and exchange that result in duplication/deficiency crossover products. Analysis of one set of such products suggests that copia and B104, though lacking any detectable homology, have undergone an exchange to produce a chimeric copia-B104 sequence at the junction of the exchange site. This unusual event is being further investigated by sequencing the junction segment.

(Continuation of Project No. Z01 ES 61011-05 LG)

PROJECT DESCRIPTION

PROBLEM: This project addresses two components of gene organization and function. The first deals with the mechanisms by which genes respond to developmental signals, while the second has to do with gene mutation and transmission.

OBJECTIVES: The objectives are (1) to characterize mutants that are modified in regulatory functions in order to identify regulatory elements and discover how they function; (2) to study the nature of spontaneous and induced gene changes and relate those changes to the mutation process and to gene organization and transmission.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: The white locus in Drosophila provides a model system for studying gene mutation, regulation and transmission. The locus is a dispensable one in the sense that a deletion is viable, though mutant, in homozygous state. There are many mutant forms of the gene and a sizable number of them perturb regulation of the locus in its developmental program. The approach is genetic and cytological analyses of white mutants coupled with the molecular cloning and in some cases sequencing of the DNA of mutant forms.

One study focuses on the mutation w^{zm} which when coupled with the mutation z^1 produces a mosaic pattern of eye pigmentation. The w^{zm} mutation is associated with the insertion of a transposable sequence of DNA, BEL, in the first intron near the 5' end of the transcribed portion of the gene and it exhibits a moderate instability. Mutant derivatives of w^{zm} show striking modifications of gene regulation, with some exhibiting mosaic patterns that are clonal and autonomous (w^{zx}), others completely lack any pigmentation (w^z), while the most unusual group shows a nonclonal, nonautonomous mosaic of pigment deposition (w^{zh}). It has been determined that the w^z mutation is the result of a second insertion of BEL DNA about 3.5kb from the site of the w^{zm} insertion. The nonclonal, nonautonomous type such as w^{zh} has lost most of both the insertion sequences in w^z ; in addition the entire white locus as well as some adjacent genes have been transposed to the left arm of chromosome 3. Of particular interest is a comparison of the mutants showing the clonal expression of the gene with those giving nonclonal action. In both there seems to be improper gene activation but this happens in some cells only. In the clonal form those cells that have proper activation, pass the activated state on through several mitotic divisions of daughter cells. In the nonclonal mutants this mitotic memory is not functioning or possibly the locus is not activated in the proper tissues, resulting in nonautonomy.

Another aspect of white locus structure being investigated is the molecular basis for intralocus asymmetrical exchanges that result in small duplication and deficiency recombinant chromosomes. Preliminary data implicate two repeated transposable DNA sequences, copia and B104. Pairing and exchange between the transposable sequences at different locations in homologous chromosomes creates duplication and deficiency products. These products are useful in characterizing regional differentiation within the locus and for studying the details of the mechanisms involved in crossing over.

A third approach to understanding white locus function is through investigation of mutants that show a chromosome-pairing dependent expression. The mutant w^{SP} produces a mosaic of pigmented cells on a very lightly colored background. It complements with most other white alleles to give a uniformly pigmented eye if the structures of the homologous X chromosomes are standard. If, however, a rearrangement is introduced into one of the chromosomes such that somatic pairing is upset, this complementation is abolished. We are examining this phenomenon to determine how the two alleles communicate with each other and how that communication is perturbed when homologs cannot pair.

RECENT ACCOMPLISHMENT AND SIGNIFICANCE: After cloning the transposon that causes the w^{ZM} mutation it has been mapped by restriction endonucleases and determined to be the same as an element found in a ribosomal RNA gene by Pellegrinis lab, and also described by Goldberg as the transposon responsible for the mutation w^a. Goldberg named the element BEL (Belshazzar) and we have adopted that nomenclature. We now know that the mRNA detected from w^{ZM} flies appears to be of normal size, which explains why in a wild-type background the phenotype is normal. When combined with the closely linked mutation z¹, however, the eye phenotype is mottled. We will investigate the effect of z¹ on mRNA production, processing, etc. as a way of determining how this transposon, known to be inserted 16 base pairs 3' from the first intron/exon splice site, upsets the expression of the gene.

Libraries of several derivatives of the w^{ZM} mutant have been constructed and have been or will be screened for white locus clones. Genetic analyses of the derivatives, including a transposition of white locus and adjoining genes into 61D of the third chromosome, show that expression of white locus is dramatically modified by z¹ in some cases but not in others. These will be molecularly characterized and their mRNA products examined.

The products of asymmetrical exchanges between homologous chromosomes have been molecularly characterized by restriction mapping and by molecularly cloning the exchange junctions in some cases. The analysis shows that at least three different classes of recombinant chromosomes can be recovered from w^a/w^{bf} heterozygotes. The transposon copia is inserted into white locus in the case of w^a and B104 accounts for w^{bf}. In one exchange that resulted in a deletion of part of the white locus, we have cloned the deletion break points and discovered that it is bordered by part of copia on one side and part of B104 on the other. The exact limits of the deleted portion have not yet been determined but we know from restriction site analysis that more than 25 kb is missing, which includes a proximal segment of white locus and more than 20 kb adjacent to it. Despite this extensive deficiency, this is a viable, albeit white-eyed genotype in the hemi- or homozygous state. Apparently a B104 element well proximal to white locus in the w^{bf} chromosome underwent exchange with the copia inserted at white in the w^a chromosome. This is very unusual because very sensitive hybridization studies indicate that these two elements do not share any homology. The elements are, however, members of the retrovirus-like transposon family. The chimeric transposon is now being sequenced in an effort to determine how the unusual exchange occurred.

The analysis of the pairing-dependent expression of the w^{SP} and other mutants of white has progressed slowly but we have, by looking at both poly A+ and poly A- RNAs from the white locus region, identified a previously overlooked species of transcribed here. We are now examining the RNA produced by complementing combinations of alleles situated in normal and in rearranged (unpaired) chromosomal configurations. The model presently favored is that the trans-effect on regulation that is upset in the unpaired chromosomes is due to a cis-acting factor (RNA?) that is generated by the white locus as a part of a regulatory pathway. Mutants that lack this factor or produce a modified one can be partially complemented by diffusion of the factor from the homologous chromosome if pairing is tight.

PUBLICATIONS

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 65037-01 LG

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transposon - mediated chromosomes instabilities in Drosophila

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. H. Judd

Chief

LG, NIEHS

J. W. Jack

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LG, NIEHS

(Continuation of Project No. Z01 ES 61027-02)

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SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A strain of Drosophila (Uc) exhibiting very high rates of spontaneous mutation, chromosome rearrangement and sterility has been shown to contain up to 65 copies per diploid set of chromosomes of the retrovirus-like transposon gypsy. Reciprocal crosses of members of this strain to flies that contain no copies of gypsy in their euchromatic chromosomal segments, produce hybrid F₁ females that show high rates of mutation associated with the mobilization and insertion of gypsy into chromosome sites previously free of the transposon. The hybrid females in turn produce offspring that are highly mutable. Examination of polytene chromosomes from F₂ and F₃ offspring of these crosses probed with ³H-labeled gypsy DNA in situ shows that gypsy is mobilized in somatic cells. This is consistent with the observation that most mutations transmitted through the germ lines appear as clusters.

F₁ males from reciprocal crosses of gypsy-free by gypsy-rich males individuals are completely stable mutationally and gypsy is not mobilized.

Particular lines of Uc spontaneously become stable. In situ examination of these lines shows a great reduction in gypsy copy number (3 - 10) and no evidence of mobility of the transposon. Attempts to define the conditions responsible for the great increase in copy number and mobility of gypsy in the original Uc strain and among offspring of crosses involving that strain continue. Thus far we have shown that other systems of hybrid dysgenesis, P-M and I-R, play no role in the mobilization of the retrovirus-like transposon.

PROJECT DESCRIPTION

PROBLEM: Systems of highly mutable genes in both eukaryotes and prokaryotes have been known for decades. The characterization of the transposable DNA sequences responsible for the unusually high mutation rates is moving forward rapidly as these elements are cloned and analyzed in molecular detail. It is still not clear, however, what the conditions are that stimulate the mobilization of particular types of transposons. Particularly interesting is understanding how retrovirus-like sequences may become activated since these are widely distributed in higher organisms, particularly mammals including humans.

OBJECTIVES: We are striving to understand the nature and function of the retrovirus-like transposon gypsy in Drosophila melanogaster which can be mobilized to a high degree in individuals of particular genotypes. We want to understand the mechanism of and the conditions for the mobilization process.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: J. Lim has described the genetical and cytological properties of a highly mutable system (Uc) in Drosophila melanogaster. We have approached the understanding of this important phenomenon by isolating and characterizing the transposable element that is responsible for the high mutation rates. We are attempting to describe fully the behavior of the element, the conditions under which it is activated and the nature of the mutational changes it imposes on genes where it inserts and/or excises.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: By cloning a cut locus mutation generated from one of Lim's crosses, we determined that the retrovirus-like transposon gypsy was in that case inserted into the cut locus. Subsequent analysis of 60 cut locus mutations generated by similar crosses showed that all except four had gypsy sequences inserted at cut. The four exceptions are lethals that may be deletions of the locus. We have now studied genetically and cytologically the mutational activities and the mobilization of gypsy in a number of crosses involving the Lim stocks. The strain he designates as Uc (unstable chromosome) contains unusually large numbers of gypsy (35 - 65 per diploid set of chromosomes) in varying positions throughout the euchromatic chromosome arms. It appears that this condition is itself sufficient to cause high rates of mutation, chromosome rearrangement and sterility due to the movement of the gypsy transposon. It is equally important to note that when females of the m strain, which has no copies of gypsy in euchromatin, are crossed to Uc males, the F₁ hybrid females are highly mutable as are their offspring. On the other hand, the F₁ males are mutationally stable and, though they inherit rather large numbers of gypsy in chromosomes from their Uc fathers, those transposons are stable in the sense that they do not increase in number nor do they change position in the chromosomes. The reciprocal cross also produces mutable hybrid females and stable males.

Unlike the other dysgenic systems described in Drosophila (the P-M and I-R systems) the mobilization of gypsy occurs during early cleavage of the embryonic cells and extends at least into larval stages as evidenced by sister nuclei in salivary gland cells sometimes having gypsy occupying different chromosomal sites. The gypsy system differs in two other ways as well. There is no

evidence that maternal factors play significant roles in controlling dysgenesis as is the case in P-M and I-R. Possibly the most significant difference is the fact that gypsy is in essence a retrovirus. Unlike most of the vertebrate retroviruses, it does not undergo maturation by donning a protein envelop. That such a retrovirus-like element can be activated to the extremely high levels of mobility as seen in the Lim system raises questions about the stability of endogenous proviruses so prevalent in vertebrates, including humans. Retroviruses are implicated in tumorigenesis and other diseases including such diseases as AIDS in mammals. It is very important that we understand the conditions that lead to increased copy number and high levels of transposition for such sequences.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Study of genetic instability is as old as the rediscovery of Mendel's principles. Yet until recently, they have been treated as rare exceptional events. Presence in all genomes of substantial amount of repetitive sequences and the wide occurrence of transposable elements discovered through recombinant DNA techniques argue otherwise. It is important to understand the destabilization process and to determine whether it is a general phenomenon that may under some conditions occur in all eukaryotic organisms including humans.

PUBLICATIONS

Lim, J.K., and Crowe, D.J.: The origin of the destabilized *Drosophila* X chromosome is in the germ cells of certain hybrid females. Genetics 107: S63 (Abstract), 1984.

LABORATORY OF MOLECULAR BIOPHYSICS

LABORATORY OF MOLECULAR BIOPHYSICS SUMMARY STATEMENT

The goals of the Laboratory of Molecular Biophysics are to develop, improve and utilize spectroscopic methods to monitor the molecular interactions that occur between environmental agents and biological systems, to develop, improve and utilize analytical methodology for specified chemical agents, and to conduct biochemical, physical organic and bio-organic studies of environmental agents and their conversion products with emphasis on biomechanism elucidation. The Laboratory is organized into five separate Workgroups: Molecular Biophysics, Mass Spectrometry, Bio-organic Chemistry, Nuclear Magnetic Resonance, and Prostaglandin Biochemistry.

MOLECULAR BIOPHYSICS

The Molecular Biophysics Program is concerned with understanding, at the molecular level, the interaction of environmental agents with target biological systems, including nucleic acids, proteins and membranes. For these studies a number of highly sophisticated spectroscopic techniques (electron spin resonance, fluorescence and absorption spectroscopy, circular dichroism and stopped flow spectrometry) are employed. Particular emphasis is placed on the detection and identification of reactive free radicals (including active oxygen species) generated from environmental chemical agents by metabolic and other processes. There is now convincing evidence that free radicals are involved in a number of pathologic conditions including chemically induced carcinogenesis, pulmonary fibrosis, methemoglobinemia, hemolytic anemia and cutaneous photosensitization.

Before free radicals can be clearly implicated in any toxic effect, the free radical species must be demonstrated to exist under appropriate biological conditions. Studies to detect, identify and quantitate free radicals generated during the metabolism of xenobiotics have continued. Benzidine has been shown to undergo oxidation by horseradish peroxidase and prostaglandin synthase to a radical cation and a charge-transfer complex formed between benzidine and its two-electron di-imine oxidation product. The di-imine is a resonance structure of the nitrenium ion, the proposed ultimate carcinogenic metabolite of aromatic amines. Horseradish peroxidase also oxidizes acetaminophen to a transient phenoxyl radical, N-acetyl-p-benzoquinoneimine, which may arylate tissue macromolecules and cause hepatic necrosis. Sulfur-centered (thiyl) radicals have been detected when either cysteine or reduced glutathione was incubated with horseradish peroxidase. Since these compounds play an important role in the structure and function of sulfhydryl-containing proteins, these oxidation reactions may modulate the biological role of cysteine and reduced glutathione.

Studies on the formation of free radical intermediates from antiparasitic drugs have continued. Trypanosoma cruzi (the agent of Chagas' disease) has been found to reduce crystal violet to a carbon-centered radical which has been implicated in the biological activity of this triarylmethane dye. The free radical intermediates, generated by polymorphonuclear leukocytes in the presence of antibody coated T. cruzi, were identified as superoxide and the hydroxyl radical. Evidence has also been obtained for the one-electron reduction of SO_2 , O_2 , nifurtimox, nitrofurantoin and metronidazole by a variety of biological systems.

Free radical metabolites, formed by both the one-electron reduction and oxidation of toxic chemicals, have been successfully detected in a variety of cells.

Light is known to interact with chemical agents in tissues, such as the skin or eyes, to produce photosensitization. The chemical agent may be endogenous (protoporphyrin in erythropoietic protoporphyria), a drug (sulfonamides, declo-mycin, chlorpromazine), a topical agent (4-aminobenzoic acid and its esters in sunscreens; halogenated salicylanilides in soaps) or an environmental agent (polycyclic aromatic hydrocarbons in coal tar; amyl esters of 2-aminobenzoic acid in printer's ink). The photosensitivity response may be one of two types, phototoxic or photoallergic. While the initial step in all forms of photosensitization must be the absorption of light by the chemical or its metabolites, the precise mechanisms of phototoxicity and photoallergy are unknown. Evidence has been sought for the involvement of free radicals and active oxygen species in photosensitization. The UV irradiation of chlorpromazine results in the homolytic cleavage of the carbon-chlorine bond to yield a highly reactive aryl free radical that readily abstracts a hydrogen atom from suitable donors.

Chlorpromazine also generates singlet oxygen (luminescence at 1268 nm) when irradiated in benzene, hexane and cyclohexane but not in aqueous or alcoholic solutions. Photodehalogenation also occurs during the UV irradiation of several antibacterial salicylanilides and the antiarrhythmic drug amiodarone to yield aryl radicals which may covalently bind to tissue macromolecules or abstract a hydrogen atom from biological substrates. The aryl radical derived from the UV-radiation induced loss of bromine from 3,4'5-tribromosalicylanilide reacted with cysteine or glutathione to give the corresponding thiyl radical.

Several advances have been made in the areas of instrumentation and/or computation. A singlet oxygen detection system, utilizing a liquid nitrogen-cooled germanium diode, has been constructed and tested. This device, which detects singlet oxygen directly by measuring its luminescence at 1268 nm, has been used to determine the efficiency of light-induced singlet oxygen production by several photosensitizers including anthracene, benzoxazole and several phenothiazine drugs. A conventional flash photolysis system has been set up and successfully interfaced to a microcomputer. Finally, a computer-aided approach to solving ESR spectra has been implemented on a minicomputer. This new program now permits the automatic matching of a computer-simulated spectrum with an experimental spectrum.

MASS SPECTROMETRY

The work of the Mass Spectrometry Group involves the application of advanced mass spectrometric methods to solve analytical problems and the development of new techniques in anticipation of future analytical requirements.

Recent work in method development has focused on three areas: development of the liquid metal ion source, progress in liquid chromatography-mass spectrometry, and utilization of tandem mass spectrometry. The potential of the liquid metal ion source as an imaging technique has been demonstrated and mass spectra of several biomolecules have been recorded. Liquid chromatography-mass spectrometry studies have provided an improved understanding of the ionization processes involved. Thermospray ionization of neutral compounds, for example, has been shown to be predominantly a gas-phase process. The use of a tandem double-focusing mass spectrometer has provided important structural data in the study

of peptides. Similar techniques applied to analysis of acyl carnitines in clinical samples have permitted the differentiation of isomeric species.

A new method for structure determination based upon endothermic ion/molecule reactions of accelerated ions with a reactive collision gas has been tested successfully: isomeric $C_2H_5O^+$ ions react differently with NH_3 at 1-10 eV kinetic energy, and the thresholds of endothermic processes agree for the most part with thermochemical predictions. Fast atom bombardment has been shown to favor species concentrated near solution surfaces, and has been applied to the analysis of silicate solution surfaces. New applications of sector-instrument collisional activation have been made, including the development of kinetic energy release data for identification of isomers at the 1 ng level and the development of theory and experiment to show that fragment ion intensities relate to thermochemistry of fragmentation even when the parent ion is not thermalized. Collisional activation studies were shown to require transmission of the entire primary beam to be usable for structure determination; further, the relation of fragment ion intensities to unattenuated main beams is required in quantitative analysis of isomers.

Approximately 700 samples have been analyzed as part of the service and collaborative support program. The techniques employed spanned the full range of mass spectrometric methods and instrumentation available to the group. Collaborative projects included applications of combined liquid chromatography-mass spectrometry, contributions to drug metabolism studies and the use of tandem mass spectrometry in structure elucidation.

NUCLEAR MAGNETIC RESONANCE

The objective of the biophysical Nuclear Magnetic Resonance (NMR) program is the elucidation of the mechanisms by which chemicals and heavy metals present in the environment cause cell injury. Specific studies carried out within the framework of this objective fall into two categories: in vivo metabolic analysis by NMR spectroscopy focused on parameters thought to be involved in the mediation of cell injury; in vitro NMR studies of the interaction of various chemicals or heavy metals with proposed or demonstrated biochemical targets.

As a consequence of the postulated role of an increase in intracellular calcium levels in the mediation of cell injury, effort has been focused on methods used to determine this parameter. Due to the relatively poor sensitivity of direct observation by ^{43}Ca NMR, an indirect method was recently developed by Feeney and coworkers utilizing a fluorinated calcium chelate - fluoroBAPTA. This chelate can be loaded into a variety of cells using a strategy initially developed for fluorescent dyes in which the chelate is administered as a neutral membrane permeable ester which then loads as a consequence of the action of cell esterases. Fluorine-19 NMR studies then allow detection of resonances from both the free and calcium complexed chelate, and hence a determination of the cellular calcium levels. Since one of the most significant limitations of fluorescent calcium sensitive dyes has been an inability to deal with cells with a large fluorescent background such as arises from hemoglobin, and with the effect of anoxia which produces large increases in the levels of fluorescent reduced pyridine nucleotides, initial investigations using the NMR sensitive chelate have focused on erythrocytes. In addition to studies with normal erythrocytes, studies on cells derived from patients with sickle cell anemia have been carried out, and the

effects of anoxia (which leads to sickling) on cell calcium levels have been determined, for the first time. In parallel with these applications, efforts are in progress to design and synthesize fluorinated calcium probes with greater sensitivity and selectivity.

As a consequence the central role of the liver in the metabolism of xenobiotics, a methodology has been developed for the analysis of hepatic metabolism of the anaesthetized rat. This approach involves the surgical removal of a portion of muscle directly over the organ followed by suturing the skin, resulting in the creation of a herniated area directly above the organ. Consequently, the metabolism of the liver can be studied using an externally placed "surface coil". This procedure is considerably less traumatic than the total "externalization" of the organ, and also has many significant advantages over the surgical implantation of an rf NMR coil. Using this approach, efforts have been initiated to analyze the effects of hepatotoxins with well established mechanisms. Efforts are now in progress to correlate the effects of an extensively studied hepatotoxin - ethionine, on several parameters which can be determined by NMR: cellular pH, ATP levels, and the concentrations of several cations. In this way, it is anticipated that in vivo data can be obtained for the sequence of events in vivo associated with the development of cell injury.

In addition to these in vivo spectroscopic studies, several in vitro studies have been carried out as well. One series of studies was designed to determine the biosynthetic origin of tabtoxin, a plant toxin produced by the microorganism *Pseudomonas syringae*. This toxin irreversibly inhibits glutamine synthetase, thus rendering the cell incapable of reassimilating the ammonia released by normal photorespiration. The tabtoxinine- β -lactam is produced from a dipeptide pretoxin containing the tabtoxinine- β -lactam and a C-terminal serine or threonine. Biosynthetic studies were carried out by using various ^{13}C enriched glucose precursors in combination with the detection of the label distribution using ^{13}C NMR. It has been determined that the tabtoxinine- β -lactam arises from the condensation of an aspartate-like four carbon unit, a two-carbon unit, and one carbon from the single carbon pool. The two carbon unit arises from carbons 2 and 3 of pyruvate.

For some time, we have carried out studies aimed at elucidating the nature of the interaction between clinically important anti-folate drugs used in the treatment of neoplastic disease, and the target enzyme dihydrofolate reductase. These studies have involved the use of specifically ^{13}C labeled inhibitors methotrexate and trimethoprin, in combination with ^{13}C NMR analysis of the inhibitor-enzyme complex. In particular, it has been determined that the enzyme-complexed inhibitors exist in the protonated form beyond pH 10, although free methotrexate has a pK of 5.7. During the past year, studies have been carried out with a mutated form of the enzyme in which the aspartate residue thought to be involved in a salt bridge interaction with the protonated inhibitor has been replaced by an asparagine residue. In this system, it was found that complexed inhibitor is unprotonated, and remains unprotonated well below the pK of the free inhibitor.

PROSTAGLANDIN BIOCHEMISTRY

The Prostaglandin Group investigates the metabolism of arachidonic acid to prostaglandins (PG), hydroxy fatty acids (HFA) and leukotrienes (LT) and studies

their role in a number of important physiological and pathophysiological events. The group also studies the metabolism of chemicals by prostaglandin synthase (co-oxidation) in order to determine the role of this metabolic pathway in the development of chemically-induced carcinogenesis.

The metabolism of aromatic amines by prostaglandin H synthase (PHS) was extensively studied. The bladder carcinogen 2-aminofluorene (2-AF) is metabolized to 2-nitrofluorene, 2-aminodifluorenylamine and azofluorene. Evidence was obtained for a free radical mechanism. We also studied the oxidation of 2-naphthylamine by PHS and horseradish peroxidase (HRP). The metabolite profile for the two enzymes was different. HRP produced metabolites similar to those found for 2-AF, while PHS produced a quinone imine metabolite. We also characterized 2-AF-DNA adducts and found a unique adduct catalyzed by PHS. A new method for isolating polar DNA-adducts was developed.

Tryptophan pyrolysis products are also excellent PHS substrates and are oxidized to mutagenic metabolites. We also observed that glutathione is oxidized to a thiyl radical by PHS and this radical reacts with aliphatic double bonds, for example styrene, to form glutathione conjugates. This represents a new mechanism for the formation of glutathione conjugates.

The role of arachidonic acid (AA) metabolism in controlling or modulating biological processes was studied. We found, using dog tracheal epithelial cells, that the biosynthesis of PGD₂ controlled the secretion of Cl⁻ ion. These cells also oxidized AA to LTC₄ and an unknown metabolite. LTC₄ also stimulated Cl⁻ secretion by stimulating AA metabolism to PGD₂. Human nasal and bronchial epithelial cells oxidized AA to metabolites derived from 15-HPETE. Identification of the metabolites is not complete. No PGs were observed and Cl⁻ secretion was very low and not controlled by AA metabolism. We also studied the effect of Vitamin K on PGI₂ biosynthesis by endothelial cells. Vitamin K did not inhibit PHS or PGI₂ synthase but did inhibit PGI₂ synthase by preventing the release of arachidonic acid in cells. Vitamin K inhibition appeared to be cell specific in that inhibition only was observed with smooth muscle or endothelial cells which are major cellular sources of PGI₂. We have also started to examine the role of AA metabolism in mitogenic responses. Mitogen response by B and T lymphocytes was inhibited by lipoxygenase or phospholipase A₂ inhibitors but not by inhibitor of prostaglandin H synthase. We intend to study this phenomenon in more detail and determine mechanism for the inhibition.

BIO-ORGANIC CHEMISTRY

The Bio-organic Chemistry program is concerned with the development of methodologies to detect and identify metabolites of environmental chemicals in biological systems. Research is also carried out on the mode of interaction of environmental agents with biological systems at the molecular level with particular emphasis on metabolic factors.

The undesirable biological effects of phthalate esters (the most ubiquitous of all environmental pollutants), which include acute testicular atrophy resulting in male sterility, hepatocarcinogenesis in rats and mice, and proliferation of peroxisomes, are mediated through their metabolites. Some twenty seven metabolites of the most widespread phthalate [di-(2-ethylhexyl)phthalate] have been identified in several mammalian species, a metabolic pathway for their formation

has been postulated, and details of the pathway elucidated through in vitro studies. As the biological activities of the different metabolites are elucidated, it becomes clear that species differences in metabolism can potentially result in the resistance of non-rodent species to the undesirable biological effects. This renders extrapolation of toxicity tests from rodents to man high unreliable.

The emphasis this year has been on the evaluation of techniques used in the study of lipid peroxidation, especially as enhanced by tumor promoters. Although it was possible to demonstrate enhancement of NADPH-dependent, iron-dependent lipid peroxidation by 2,3,7,8-TCDD in vitro, it became clear that the majority of the techniques commonly used for such studies can often produce misleading results. Moreover, the more commonly used "model systems" are in fact so chemically complex that results from their use can only be interpreted by making a number of untested or inadequately tested assumptions. Therefore we are attempting to develop more reliable and quantitatively interpretable assays for measuring (not just monitoring) lipid peroxidation, investigating the validity of commonly applied assumptions as to the function and role of various components of model systems, and examining the oxidized products of such systems. Once a repertoire of validated methodologies has been established, studies of the enhancement of lipid peroxidation in vivo and in vitro will continue.

Other research has focused on the description of mechanisms at various biochemical and molecular levels including development of structure-activity correlations as a predictive tool in toxicology. The role of thyroid hormone binding proteins in mediating the toxic effects of certain halogenated aromatic hydrocarbons of environmental importance is being investigated. Current interest is in the binding proteins specific for thyroxine (T₄). Prealbumin (TBPA) is a major thyroxine binding protein in blood which has been proposed as a model for the thyroxine nuclear receptor in tissue. Molecular interactions of TBPA with the dioxins, furans and polychlorinated biphenyls (PCBs) have been studied with use of computer graphics and predictions made regarding relative binding affinities for such structures. These modeling predictions were tested by experimentally measuring the binding affinities of soluble derivatives of those structures, and the results are in good agreement with prediction. The binding model can account for the requirement for lateral halogens and an approximately rectangular molecular shape in toxicity. Similar studies with the thyroxine nuclear protein solubilized from rat liver tissue also show competitive binding interactions and with similar binding specificities. A soluble dioxin approximate isostere shows a remarkably high affinity (half-maximal of 15 pM) for the nuclear receptor (compared to a half maximal of 15 nM for T₄). Thus the nuclear receptor affinity has the expected sensitivity for possible involvement in toxicity. Dose-dependent regulation (increase) of the T₄ nuclear receptor number by dioxin was demonstrated suggesting a possible mechanism for potent and persistent expression of thyroid hormone activity which could result in toxicity.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and thyroxine have common molecular reactivity properties which enable them to present a planar face and lateral halogens in interactions with proteins. These molecular properties are consistent with the structure-toxicity relationship of TCDD and related compounds. Biological evidence is accumulating, including preliminary studies on the effects of TCDD exposure on tadpole growth and development and on murine stem cell proliferation and differentiation, which is consistent with the possible

thyroxine-like activity of TCDD. The work suggests the possibility that toxicity is at least in part the expression of potent and persistent thyroid hormone activity (responses induced by TCDD which qualitatively correspond to those mediated by thyroid hormones). A mechanism for toxicity has been proposed which involves receptor proteins; the planar aromatic system controls binding to cytosolic proteins and halogen substituents regulate binding to nuclear proteins. This simple model based on molecular reactivity sheds light on the diversified effects of TCDD and related compound toxicity and on certain thyroid hormone action. The model also permits predictions to be made with regard to the toxicity and thyroid hormone activity of untested compounds.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 10003-06 LMB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Synthetic and Analytical Studies in Bioorganic Chemistry		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Oscar Hernandez	Senior Staff Fellow	LMB NIEHS
OTHER: M.B. Gopinathan Michael Walker	Visiting Fellow Chemist	LMB NIEHS LMB NIEHS
COOPERATING UNITS (if any) Laboratory of Behavioral and Neurological Toxicology Development and Reproductive Toxicology		
LAB/BRANCH Laboratory of Molecular Biophysics		
SECTION Bio Organic Chemistry		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 0	PROFESSIONAL: 0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The aim of this project is to develop chemical methods and apply these to specific biological areas. Two problems of current interest are 1) chemistry of glutathione and its adducts with epoxides; 2) mechanisms of separation and purification of polypeptides by high-performance liquid chromatography. The level of success of a program on bio-mechanisms, such the one posed by the glutathione pathway, is largely dependent on the ability to generate the chemical information necessary for the interpretation of biological experiments. A systematic program was initiated and eventually has culminated in the development of unequivocal syntheses of the requisite compounds, methods of separation for diastereoisomeric conjugates of glutathione with alkene and arene oxides, establishment of the absolute configuration of these thioether stereoisomers, and correlation of synthetic compounds with enzymatically produced conjugates. The high-performance liquid chromatography analysis of peptides has focused on the role of hydrophobicity in the separation mechanism by reversal phase liquid chromatography. Experiments have been designed and implemented which alter the intrinsic hydrophobicity of peptide molecules during chromatographic analysis. These changes are introduced in a predictable fashion such that valuable structural information is derived from conventional reversed-phase experiments. Successful applications of this approach include: characterization of amphibian peptides in small-cell carcinoma, purification of a protein seemingly synthesized in response to estrogen exposure, and characterization of two forms of epidermal growth factor isolated from mouse submaxillary gland.		

A. Synthetic and Analytical Studies in Bioorganic Chemistry

Nature of Problem: The level of success of a program in biomechanisms is highly dependent on the ability to generate the chemical information necessary for the interpretation of biological experiments. Because of this established symbiosis, the nature of this information is defined by the particular needs of the project at hand. In general, this program does not deal with the implementation of published procedures but rather with the development of synthetic and analytical methodology and with the application of these methods to specific biological problems. This project in its inception reflects the interactive nature of chemistry whereby ideas and knowledge originate in response to challenges for biological disciplines. Two areas of current interest are: 1) chemistry of glutathione and its adducts with epoxides; 2) mechanisms of separation and purification of peptides by reversed-phase high-performance liquid chromatography.

Objectives: The principal objectives in the glutathione project are 1) to design unequivocal synthetic routes leading to glutathione adducts of alkene and arene oxides; 2) to develop chromatographic procedures for the separation and purification of stereoisomeric glutathione conjugates of epoxides; 3) to establish the absolute configuration of diastereoisomeric glutathione conjugates produced in rats. The steps outlined above were implemented in order to ensure the chemical and stereochemical integrity of the synthetic samples. Based on this knowledge, meaningful correlations with enzymatic systems could be subsequently attempted.

For the peptide portion of the project, the focus was on the role of hydrophobicity in the separation mechanism of peptides by reversed-phase high-performance liquid chromatography. The hypothesis tested in this segment was that the disruption of the hydrophobic surface accessible for binding of a peptide molecule to a reversed-phase bonded column would result in increased or decreased retention in the chromatographic column, and that the direction of this change could be predicted based on the nature of the disruption introduced. The immediate benefit available from this approach would be increased selectivity in peptide separations; subsequently, the predictive value of these experiments could be extended to structural studies of peptides where the amounts of sample available severely limit the application of conventional techniques.

Experimental Approach: For the synthesis of glutathione adducts of epoxides two design features were particularly important. First, it was critical that the conditions used would not result in racemization of the resulting thioether conjugates. Second, that the anticipated mixtures should be produced in a form suitable for chromatographic purification. The first consideration required the initial development of a method to assess the extent of racemization of the peptide moiety, and based on these experiments a suitable base and solvent combination could be selected. The second consideration could be satisfied by using normal-phase chromatography, which would provide the capacity required for purification of relatively large sampler. This latter feature also dictated the use of a fully-protected, organic solvent soluble glutathione.

For the modification of selectivity during reversed-phase column chromatography of peptides, it became evident that pH was the logical variable to introduce

changes designed to alter the intrinsic hydrophobicity of peptide surfaces. For small peptides, up to fifteen amino acid residues, our initial efforts were concentrated on the presence of histidine residues as a way of altering hydrophobicity as a function of pH. At low pH the imidazole ring would be protonated thus disrupting the hydrophobic surface accessible for binding. At neutral pH this binding surface on the peptide molecule would be reestablished with a concomitant, and predictable, effect on column retention. For larger peptides, where conformational effects grow in importance, the effect of pH on selectivity was also deemed important based on the following argument. At neutral pH a number of amino acid residues participate in hydrogen bonding, salt bridges, etc. The combination of these interactions results in a preferred conformation(s). If, however, the pH of the solvent is lowered a substantial portion of these intramolecular interactions would be inhibited resulting in the adoption of a conformation different from that at neutral pH. The conformers obtained at acid and neutral pH would more likely differ in the nature and extent of hydrophobic surfaces accessible for binding and this in turn would result in increased selectivity during reversed-phase liquid chromatography. This concept has been validated by the separation and purification of two forms of mouse epidermal growth-factor.

Proposed Course: No further work will be done on the project.

C. Publications of past 18 months:

Hernandez, O., Dermott, K., and Lazarus, L.: High-performance liquid chromatography of amphibian peptides. Selectivity changes induced by pH. J. Liquid Chromatogr. 7: 893-905, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 10004-06 LMB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies in Nuclear Magnetic Resonance (NMR) Spectroscopy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Robert E. London	Research Physicist LMB NIEHS
OTHER:	Louis Levy	Research Chemist LMB NIEHS
	Elizabeth Murphy	Staff Fellow LMB NIEHS
	C. Tyler Burt	Expert LMB NIEHS
	Ronnie R. Rippy	Electrical Engineer LMB NIEHS
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COOPERATING UNITS (if any)		
LAB/BRANCH		
Laboratory of Molecular Biophysics		
SECTION		
Nuclear Magnetic Resonance Group		
INSTITUTE AND LOCATION		
NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
5.2	3.2	2.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>The objective of the biophysical <u>nuclear magnetic resonance (NMR)</u> program is the elucidation of the mechanisms by which chemicals and heavy metals present in the environment cause <u>cell injury</u>. The development and application of NMR methodology in order to achieve this objective may be considered to fall into two broad categories: 1) In <u>in vivo</u> <u>metabolic analysis</u> using NMR spectroscopy. Such studies probe directly the <u>metabolism</u> of various xenobiotics when sufficient concentrations are present to permit detection. Additionally, studies of the effects of these agents on metabolic parameters thought to play an important role in the mediation of cell injury are carried out. In addition to measurements of intracellular pH and levels of high energy phosphate compounds, current emphasis is on the measurement of free intracellular <u>calcium levels</u> and on the development of <u>fluorinated NMR active spin traps</u> for the <u>in vivo</u> detection of intracellular <u>free radicals</u>. Cellular calcium levels are measured using a fluorinated calcium chelate in combination with ¹⁹F NMR detection. Measurements have been carried out in a variety of cell and perfused organ systems, with current emphasis on red blood cells in which fluorescent calcium sensitive dyes cannot readily be used. Efforts are in progress to develop more specific and sensitive probes for calcium and other cellular cations. 2) <u>In vitro</u> studies of the interaction of various chemicals with known or proposed biological targets. Recent studies have involved the use of ¹³C labeled antifolate drugs, particularly [2-¹³C] methotrexate, with the enzyme <u>dihydrofolate reductase</u>. NMR studies of several flavoenzymes including <u>xanthine oxidase</u>, <u>sulfite oxidase</u>, and <u>glucose oxidase</u>, are also being carried out.</p>		

A. Studies in Nuclear Magnetic Resonance (NMR) Spectroscopy

Nature of Problem: One of the principal limitations on attempts to elucidate the mechanisms of chemical toxicity at the molecular level is the need to deal with isolated cellular components. Indeed, the reductionist strategies of traditional biochemical analysis which have proven so fruitful are all eventually limited to extrapolation from an in vitro model. This extrapolation may be most misleading if the toxic response involves primarily a perturbation of the regulation of cellular metabolism, since it is particularly difficult to obtain meaningful regulatory data with in vitro or even in situ systems. Additionally, perturbations of various aspects of electrolyte balance of cells may represent an important component of the toxic response. During the past decade, an alternative NMR approach has been developed which permits the analysis of cellular metabolism in vivo and can be extended to intact experimental animals. There are some significant limitations of this technique at present - particularly the limit of sensitivity which restricts studies to relatively concentrated metabolites. Nevertheless, in certain instances the use of specific indicator molecules such as the calcium specific chelate discussed below, can extend the sensitivity into the submicromolar range. The development of non-invasive probes for the evaluation of the metabolic response to toxic chemicals could evolve into a significant tool of toxicological analysis, leading in particular to a more efficient use of experimental animals in such studies.

Although in principle the molecular basis of cell injury can be as varied as the structure of chemical toxins which are studied, several common pathways have been found to be significant in a wide variety of cases. These include: the reduction in cell ATP levels with the associated impairment of all aspects of energy requiring metabolism; alterations in the levels of all intracellular electrolytes, particularly calcium; the production of free radicals either directly as metabolites of xenobiotics, or indirectly via stimulation of the production from endogenous sources to yield species such as superoxide. Recent developments in magnetic resonance spectroscopy offer the potential for evaluation of all of these parameters and for correlating the response to any particular xenobiotic. In vivo ^{31}P NMR studies have now become routine and offer the possibility of obtaining information on ATP levels as well as cellular pH based on the chemical shift of molecules such as inorganic phosphate with pK values near 7.0.

The limited sensitivity of the NMR technique has been the principal drawback of this technique in the study of biological systems. Many of the cellular metabolites of greatest interest are present at levels well below detection threshold. Of these, the level of free cellular calcium is one of the most significant, particularly in terms of its role in the mediation of cell injury. Recently Tsien has developed a fluorescent dye, quin2, which permits the quantitation of free intracellular calcium levels, and an analogous NMR probe has also been tested. In the latter case, the probe is a chelate which contains a fluorine nucleus in order to allow detection by ^{19}F NMR. The chelate is administered to the cells under study as a tetra-acetoxymethyl ester which is neutral and hence can penetrate the cell membrane. Cell esterases hydrolyze the ester so that the cells "trap" and concentrate the chelate. The sensitivity for detection of the

calcium ion is then based on the association constant between the free cellular calcium and the chelate. After equilibrium is reached, changes in free cellular calcium levels can readily be measured. The NMR active calcium probe has several significant advantages compared with fluorescence measurements. Additionally, effects of conditions such as anoxia can be studied easily without interference due to fluorescence from reduced pyridine nucleotides [NAD(P)H].

For the third parameter noted above, the sensitivity of NMR observables to the presence of free radicals is relatively non-specific - most generally reflecting changes in relaxation parameters due to the dipolar interaction of the radicals with the nuclear spins. In contrast, electron spin resonance (ESR) techniques have proven to be more useful for the identification and quantitation of free radicals. Much of the progress made in this area in recent years has been based on the use of spin traps - reactive nitrones or nitroso compounds which form stable spin adducts with free radicals which are formed by various mechanisms:



As a consequence of the stability of these nitroxide adducts, they can be readily detected and analyzed in order to provide information on the nature of the radicals (R[•]) formed in the cells.

Despite the great utility of this technique, applications have generally focused on model systems as a consequence of the reducing environment which exists in the cell. Thus, ascorbate will reduce the nitroxides to give the corresponding non-paramagnetic hydroxyl amines which cannot be detected by ESR. In principle, these reduced products could be readily detected by NMR, but a significant limitation would be the problem of determining which of the enormous number of proton or carbon resonances of the intact cell correspond to the reduced spin adduct. A potential solution to this problem is the use of a spin trap with an NMR tag - in this case, a fluorine nucleus. As a consequence of the lack of naturally occurring fluorine in cells, as well as its relatively excellent NMR sensitivity, this nucleus makes an ideal label for NMR studies. Hence, the use of a fluorinated spin trap offers the potential for the *in vivo* detection of cellular free radicals. Such traps should prove useful in providing the same information generally obtained in an ESR study, plus having the additional NMR handle which would permit NMR analysis of the adducts.

In addition to *in vivo* metabolic analysis, NMR spectroscopy remains a powerful tool for *in vitro* studies generally, and for the evaluation of the interactions of chemicals with biological molecules in particular. The NMR group carries out a broad range of studies in order to elucidate the conformations of environmentally important chemicals in general, and the interactions of these chemicals with proposed biological target tissues. Principal among these has been a continuing series of studies of the interaction of various clinically important anti-folate drugs such as methotrexate and trimethoprim, with the target enzyme

dihydrofolate reductase. Recent studies carried out in collaboration with Dr. R.L. Blakley of St. Jude Children's Research Hospital in Memphis, have focused on the nature of the interaction of inhibitors labeled with carbon-13, with the aspartic acid residue (Asp-27) at the active site. The combination of specific labeling and NMR detection allows quantitation of particular interactions of interest in highly complex systems such as the enzyme - inhibitor complex. Finally, as part of these programs, synthetic effort is directed toward the production of labeled molecules of interest (typically involving a ^{13}C label) as well as the synthesis of other molecular probes such as the fluorinated calcium probe and spin traps discussed above.

Experimental Approach: The objectives of this activity include: (1) development of NMR methodologies for monitoring metabolic function generally, with emphasis on parameters thought to be important in the mediation of cell injury; (2) comparison of data for isolated cells, perfused organs, and intact experimental animals; (3) correlation of the effects of various chemicals of interest on the different parameters. Methodological developments include the design and construction of in vivo NMR probes used for the analysis of the metabolism of perfused organs and intact experimental animals, and the design and synthesis of chemical probes such as the fluorinated spin traps and calcium indicators discussed above. Subsequently, these chemical probes must be evaluated in each cell type of interest from the standpoints of efficacy of the measurement, chemical toxicity, optimization of NMR pulsing parameters, and optimization of loading levels to obtain data in the shortest amount of time. Ultimately, the objective is to simultaneously or concurrently obtain data on a broad range of cellular parameters including levels of various cations including calcium, magnesium, sodium and potassium; cellular pH, ATP levels and cellular free radicals as reflected by the fluorinated spin traps under development.

Recent Accomplishments and Future Plans: The advent of surface coil methodology has made it possible to obtain spectroscopic data from intact experimental animals and humans. A large fraction of the studies carried out to date has involved muscle physiology and metabolism as a consequence of the proximity of this tissue to the surface of the organism. Some data have been obtained from internal organs using a variety of approaches which involve either sophisticated localization by the use of complex pulse sequences or field gradients, or the simpler use of surgical strategies such as a direct exposure of the organ to the surface coil or the implantation of the surface coil. The technological approaches noted above are ideal from the standpoint of providing a truly non-invasive measurement but suffer from a variety of practical limitations which include the need for some very sophisticated equipment, low sensitivity reflecting an intrinsically poor filling factor, and some limitations in coverage of the frequency spectrum required for the measurement. We have recently developed a simpler approach involving the removal of the layer of muscle directly over the organ to be studied followed by suturing of the skin. The herniated region thus produced heals rapidly and the rat can then be studied over a long period of time for the evaluation of the response to various toxic substances. Using a surface coil constructed for the Nicolet NT-360 spectrometer, we have carried out a variety of studies of the hepatic metabolism of the intact rat by phosphorus, carbon, and fluorine NMR.

Effects of Agents which Selectively Lower Cellular ATP Levels: There exist several hypotheses explaining how various chemical agents can produce cell injury. Some of the principal theories include: 1) injury resulting from lowered ATP levels with the consequent reduction in all energy requiring activities; 2) injury resulting from the accumulation of cellular calcium with the accompanying errors in all cellular processes which are calcium regulated; 3) injury resulting from highly reactive free radicals formed in response to chemical agents. In order to obtain a better understanding of the effects of each mechanism it is useful to introduce perturbations of each of these parameters individually and to assess the impact on all cellular parameters which can be determined by this method. We have utilized two strategies which will deplete cellular ATP levels: 1) The introduction of high levels of fructose leads to a rapid depletion of both ATP and inorganic phosphate levels in the liver as a consequence of the rapid formation of fructose-1-phosphate by fructokinase. Cellular ATP levels reach a minimum approximately five minutes after the introduction of the fructose. There is subsequently a rapid increase in ATP, but the levels do not return to the preadministration level, presumably as a consequence of further catabolism of the AMP formed to IMP and other purine catabolites. The subsequent gradual return of the level to the control values is limited by the *de novo* synthesis of the purine base. We have studied this phenomenon in the Tiver of the intact rat, and subsequently will be correlating changes in the ATP levels with changes in the levels of cellular pH and the levels of other cations, particularly calcium. 2) A second well established means of lowering cellular ATP involves the administration of the ethyl analog of methionine: ethionine. The subsequent biosynthesis of S-adenosyl ethionine ties up a large fraction of the purine base, again lowering cell ATP levels. This approach has the advantage of producing a more sustained decrease in the cell ATP levels, but also introduces additional perturbation which will lead to cell injury over a longer term - alkylation with the ethyl group, and interference with methyl group metabolism. As in the example of fructose, it will be of interest to determine how the ATP lowering capacities of these agents correlate with changes in other cellular parameters.

Measurement of Cell Calcium Levels with Fluorinated Indicators: The introduction of the fluorescent indicator quin2 for free cellular calcium has revolutionized our ability to quantitatively assess changes in the levels of this important intracellular metabolic regulator. As with any other technique, there are some drawbacks to the fluorescent dye which include: a) inapplicability to cells containing hemoglobin due to interference with absorption and fluorescence; b) quenching of the fluorescence by other intracellular ions including zinc and manganese; c) limited sensitivity due to background fluorescence of reduced pyridine nucleotides. We have recently begun work with an NMR active dye - fluoroBAPTA (FBAPTA), which works analogously to the quin 2 but is detected by fluorine-19 NMR rather than fluorescence. The dye was initially synthesized in our group as a tetramethyl ester. Unfortunately, this form of the dye did not effectively load into the cells, and the acetoxymethyl ester form was subsequently obtained from Molecular Probes, Inc. This molecule has been loaded in erythrocytes and isolated heart cells, but, to date, efforts to load liver cells have resulted in death of the liver cells. Phosphorus NMR studies indicate a rapid drop in both pH and ATP levels of the liver cells upon exposure to the dye

- indeed, the FBAPTA is the most potent hepatotoxin we have worked with. Nevertheless, it has been possible to obtain data from both normal erythrocytes and erythrocytes from patients with sickle cell anemia, and to compare the cellular calcium levels.

One of the principal experimental difficulties in the use of both fluorescent and NMR active cation dyes is the need to correct for extracellular dye which has leaked out of the cells after loading. One approach generally used is the addition of another chelate, EGTA, which will complex with extracellular calcium. Although this is effective with the fluorescent dye, the addition of EGTA will result in the creation of free FBAPTA which will be detected by NMR and interfere with the quantitation of the uncomplexed chelate. Another approach used for the fluorescent dye, addition of manganese, also interferes with the NMR measurement due to broadening of all cellular resonances by the paramagnetic ion. We have recently developed an alternative approach, addition of lanthanide ions which leads to a discrete resonance corresponding to ion complexed FBAPTA. Of the ions studied to date, Europium appears to be the most satisfactory. The Eu-FBAPTA resonance will not interfere with the quantitation of the free and calcium bound peaks in the cell and appears to be a substantial improvement over the methods currently in use.

Studies of the Interaction of Dihydrofolate Reductase with [2-¹³C] Methotrexate
As part of a continuing series of collaborative studies with Dr. R.L. Blakley of St. Jude Children's Research Hospital on the nature of the interaction between clinically used antifolates with the target enzyme dihydrofolate reductase, we have carried out a series of studies with specifically carbon-13 enriched inhibitors. These studies have shown that the enzyme complexed form of the drug is protonated, and remains protonated past pH 10.0. In contrast, the free methotrexate has a pK of 5.7. This perturbation of the pK is thought to represent an important aspect of the binding potency of the inhibitor, and to result from the formation of a salt bridge with the aspartate-27 residue on the enzyme. During the past year, we obtained enzyme from *E. coli* in which the Asp-27 residue is replaced by an asparagine residue, and we have carried out ¹³C NMR studies on this mutated enzyme using the [2-¹³C] labeled methotrexate. These studies demonstrate that, in contrast to results with the native enzyme, the enzyme complexed form is unprotonated. Further, we can reduce the pH to approximately 4.0 (below this pH the enzyme precipitates), and find that while any methotrexate free in solution becomes protonated, the enzyme complexed form remains unprotonated. The results demonstrate that the salt bridge which has been thought to be of principal importance to ensure binding of the inhibitor, is not essential for tight binding. These data have important implications for the design of specific inhibitors of this enzyme.

Evaluation of Fluorinated Spin Traps for NMR Studies of Free Radicals Initial efforts toward the development of NMR active spin traps have focused on analogs of phenyl butyl nitron (PBN) with a trifluoromethyl group at position 2 of the phenyl ring, and we are currently synthesizing a trap with two fluorine nuclei at positions 2 and 6. Preliminary studies have focused on the use of well characterized radical precursors. Studies in which photochemical activation processes were used to generate the radicals indicated that PBN rapidly isomerizes to an oxazirane. This isomerization may well alter the subsequent chemistry of the trap.

Biosynthesis of Tabtoxin Tabtoxinine- β -lactam is an irreversible inhibitor of glutamine synthetase that is produced by several pathovars of the plant pathogen *Pseudomonas syringae*. The toxin can infect and cause chlorosis in a number of crop plants including soybeans, peas, oats, and tobacco. We have examined the pathway for the synthesis of tabtoxin by using specifically ^{13}C enriched glucose precursors and determining the labeling pattern in the synthesized product by carbon-13 NMR. Successful analysis of the biosynthetic pathway required an initial determination of the pathway by which glucose is metabolized by the *P. syringae*. This was determined to be via the 6-phosphogluconate or Entner-Doudoroff pathway, as reflected by the determination of 2 keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase) activity. Using this information and the observed ^{13}C distribution in the tabtoxin, the pathway was shown to reflect a condensation of an aspartate-like four carbon unit, carbons 2 and 3 of pyruvate, and carbon 8 from the single carbon pool.

B. Plans for the Subsequent Year

Acquisition of two Large Bore NMR Spectrometers The evolution of NMR technology from a primary role as analytical chemical tool to its application as a biological tool has been correlated with the development of large bore magnets which permit the observation of intact experimental animals, and potentially of more dilute solutions and gases. Additionally, it is anticipated that it may be possible to combine different types of detection, such as fluorescence measurements, with NMR determination readily using the large bore magnets. Hence, the NMR workgroup has recently acquired two 4.7 tesla, 33 cm horizontal bore magnets in order to extend the NMR capabilities of the workgroup and the Institute as a whole. It is anticipated that one of the magnets will be used with an existing (Nicolet) data system to obtain more conventional spectroscopic data from large or dilute samples and from experimental animals. The horizontal orientation of the magnet bore also provides a less physiologically stressed position for experimental animals. The second magnet will be part of a General Electric CSI system capable of performing both spectroscopic and imaging studies. The principal advantage of this capability will be the use of gradients to obtain localized spectroscopic data. In addition to the possibility of obtaining localized spectroscopic data directly, Seelig and coworkers have recently demonstrated the use of this technique in order to localize proton decoupling to a defined region of interest, and in this way to obtain localized carbon-13 spectral data.

Development and Evaluation of Calcium Sensitive Molecular Probes Preliminary studies with FBAPTA, the fluorinated NMR probe which provides information on the free cellular calcium levels based on the fluorine chemical shift, have met with some success, but several potential improvements in this technique would greatly enhance its applicability and will be evaluated over the coming year.

1. One of the principal limitations of the best indicator, 5-fluorobAPTA which we have used is the relatively low binding constant (700 nM) for calcium. As a consequence, the resonance corresponding to the calcium complexed dye is relatively small. At typical cellular calcium concentrations, the intensity ratio of the calcium bound to the free FBAPTA will be approximately 1:10. It would be most useful to have a molecular probe with a somewhat higher calcium affinity in

order to obtain ratios closer to 1:1 for typical free cellular calcium concentrations. This would lead to a significant reduction in the time required to make the measurement which is now sensitivity limited by the need to detect the low intensity resonance of the calcium complexed chelate. Another important aspect of the affinity of the probe is the broadening of the resonance due to chemical exchange. Although the fluorine resonances of the free and calcium complexed BAPTA are in slow exchange, there is significant exchange broadening, as is readily demonstrated *in vitro*. Measurements of the spin lattice relaxation rates for the fluorine nuclei *in vivo* indicate that the T_1 is roughly 200 ms. Assuming that the motion of the molecule corresponds to the extreme narrowing limit, in which case $T_1 \approx T_2$ for most relaxation mechanisms, the broad intracellular linewidths which we have observed reflect chemical exchange - either between the free and calcium complexed forms of the dye, or as a consequence of reversible binding interactions with other cellular components. The development of a BAPTA analog with a higher affinity for calcium should decrease the exchange rate between free and calcium complexed species and reduce the first source of broadening. Since the fluorescent dye quin2 has a stronger binding constant, several analogs closely related to this structure, i.e. containing a quinoline ring, will be synthesized and evaluated. An additional strategy worth considering is the synthesis of BAPTA and analogs containing a CF_3 group rather than a directly bonded F. As a consequence of the increased number of fluorine nuclei and the increased degree of intramolecular diffusion about the CF_3 axis, enhanced sensitivity and a narrower linewidth would be predicted. This should particularly be significant if the primary source of broadening of the fluorine resonances is association with cellular components. Alternatively, it must be noted that it is difficult to predict whether the CF_3 group in which the fluorine nuclei are further removed from the ring will show sufficient chemical shift sensitivity to calcium complexation.

2. Alternative strategies for cellular loading. The loading of the BAPTA dye is currently achieved through the use of the easily hydrolyzed acetoxymethyl ester form of the molecule. This approach relies on cell esterases to cleave the acetoxymethyl group, but results in the release of eight equivalents of acid and four equivalents of formaldehyde per molecule of BAPTA. Thus, hydrolysis of the ester groups can have some significant toxic effects. As noted above, all attempts to date to load hepatic cells with the fluoroBAPTA have resulted in rapid cell death. This contrasts to results with quin2 which is hydrolyzed more slowly and which can be successfully loaded into the liver cells. The fluorinated quin2 analog noted above may prove to be less toxic. Alternatively, other loading approaches may be preferable. Recent progress has been made in the use of liposomes to deliver drugs to cells of various types, and such liposomes might also serve as vehicles for the introduction of free (non-esterified) BAPTA into cells. The use of other blocking groups, such as peptide rather than ester functions, is currently under evaluation.

Extension of the Use of Intracellular Cationic Probes to Quantitate Other Intracellular Ions In addition to the studies of intracellular calcium discussed above, there is interest in the quantitation of other intracellular cations including heavy metals such as Cd(II) and Hg(II), as well as potassium

and sodium. We have carried out preliminary studies with the fluoroBAPTA dye and found that cadmium ions are in slow exchange with the chelate and the chemical shift is diagnostic for this ion. Similarly, zinc and strontium complexes give diagnostic shifts, although in the latter case the exchange is sufficiently rapid to yield a rather broad fluorine resonance. It should be noted that while this approach may be useful for the study of intracellular pools of these metals, the approach is of somewhat limited value since, in contrast to the case of calcium, there are not large reservoirs of bound intracellular ions to replace the metals chelated by the BAPTA. Hence, introduction of the indicator will strongly perturb the intracellular free ion concentrations.

Monovalent cations such as sodium and potassium have recently been observed directly by NMR in a variety of biological systems. The central experimental limitation on such measurements is the need to introduce agents which will allow differentiation of the intra and extracellular pools. Recently several cationic shift reagents have been developed for this purpose. These approaches have several drawbacks, including extensive broadening of the resonance and shift of resonances corresponding to some of the ions not directly accessible to the reagents via bulk susceptibility effects. In view of these problems, it appears that studies of the changes cellular potassium levels in the liver of the intact rat may prove most feasible. Since the ratio of intra to extracellular potassium is high, and since the level of blood potassium must be closely regulated to maintain cardiac function, it should be possible to monitor changes in the potassium levels in the liver of the intact rat in response to various perturbations using the assumption that extracellular contributions to the resonance will be relatively small and constant.

³¹P NMR Studies of Xanthine, Sulfite, and Glucose Oxidase We have recently initiated collaborative studies on the ³¹P NMR of several oxidases with Drs. Jean Johnson and K.V. Rajagopalan of Duke University. These enzymes have been of particular interest from a toxicological standpoint in several respects: (1) Xanthine oxidase has been proposed to play an important role in so called "reperfusion injury". According to this theory, ischemic periods are associated with an accumulation of xanthine and hypoxanthine coupled with the conversion of xanthine dehydrogenase into the oxidase form. Subsequent oxidation of these metabolites upon return of the oxygen supply results in the associated generation of oxygen free radicals. (2) Sulfite oxidase is a molybdohemoprotein which catalyzes the terminal step in the degradation of sulfur amino acids. In addition to this role in detoxification of endogenously derived SO₃²⁻, the enzyme is an important defense against exogenous bisulfite and SO₂. Previous studies have indicated that rats made deficient in sulfite oxidase activity by the administration of dietary tungsten (which competes with molybdenum uptake and utilization) are more susceptible to the deleterious effects of injected bisulfite and respired SO₂. These enzymes, as well as several additional oxidases, have recently been studied by ³¹P NMR by D.E. Edmondson and coworkers, and in each case, new phosphorylated residues have been identified. In the case of glucose oxidase, the spectra were interpreted as indicating the presence of a phosphodiester linkage in the protein.

Drs. Johnson and Rajagopalan have not found biochemical evidence for the additional phosphorylated residues in any of these systems, and our preliminary NMR results are not in agreement with the data of Edmondson and coworkers. Studies carried out to date suggest that the additional phosphate resonances observed arise from degradation products of the flavin cofactor of these enzymes, and further work is in progress to analyze this effect.

¹³C NMR Studies of Dihydrofolate Reductase As noted above, studies during the past year have involved the use of [2-¹³C] methotrexate and the analysis of its interaction with *E. coli* dihydrofolate reductase (DHFR), and with a mutated form of the enzyme in which an asparagine residue has been substituted for the aspartate at position 27. Another approach which we have followed in NMR investigations of DHFR involves the incorporation of ¹³C enriched amino acids into the enzyme by growth of a microorganism on medium containing the labeled amino acids. In this way, [guanidino-¹³C] arginine, [methyl-¹³C] methionine, and [γ -¹³C] tryptophan labeled DHFR derived from *S. faecium* have been produced. One of the principal limitations of this approach is the problem of assigning the enzyme resonances. Thus, there are eight arginine residues, seven methionines, and four tryptophans in the *S. faecium* enzyme and assignment within these groups can prove extremely difficult. One approach which we have developed involves the use of spin labeled analogs of the cofactor, NADPH, or the inhibitor, methotrexate, which will broaden specifically the ¹³C resonances corresponding to residues nearest the binding sites. This approach will be extended during the next year in order to try to complete the assignment of the tryptophan labeled enzyme.

Development of Fluorinated Spin Traps for NMR Analysis As noted above, we are currently in the process of evaluating several fluorinated spin traps, particularly o-CF₃-PBN, in order to study the formation of free radicals in intact tissue. In order to carry out these studies, it is now necessary to apply these NMR spin traps to several simple chemical systems. Principal effort will be focused on phenyl radicals generated by decomposition of phenylazotriphenylmethane. Preliminary studies indicate that exposure of the fluorinated trap to this reagent yields principally one product which is, however, not simply the phenyl nitroxide adduct. Efforts to further characterize this product are in progress. Several other sources of both phenyl and alkyl radicals will be investigated during the coming year.

C. Publications of Past 18 Months

Bobenreith, M.J., Levy, L.A., and Hass, J.R.: A study of the electron impact induced retro diels-alder process with selected 5,5,6a,7,12,12a hexahydrobenzanthracenes. Organic Mass. Spectrom. 19: 153-158, 1984.

Scott, L.T., Tsang, T.H., and Levy, L.A.: Automerizations in benzenoid hydrocarbons. New mechanistic insights from the thermal rearrangements of benzo(a)anthracene-5-¹³C. Tetrahedron Lett. 25: 1661-1664, 1984.

Levy, L.A. and Sushikumar, V.P.: The synthesis of chrysene, 5-substituted chrysenes and chrysene derivatives via intramolecular cycloaddition reactions. J. Org. Chem. in press.

Parker, C.E., Levy, L.A., Smith, R.W., Yamaguchi, K., Korach, K.S., and Gaskell: S.J. separation and detection of enantiomers of stilbestrol analogues. J. Chromatog. (in press).

London, R.E., Galvin, J.J., Thompson, M., Jeffreys, L., and Mester, T.: An approach to NMR studies of the metabolism of internal organs using surface coils. J. Biochem. Biophys. Methods 11: 21-30, 1985.

London, R.E.: Carbon-13 labeling strategies in macromolecular studies: Application to dihydrofolate reductase. In G.C. Levy (ed). Topics in Carbon-13 NMR Spectroscopy, Wiley and Sons, 1984, pp. 53-90.

London, R.E. and Walker, T.E.: Biosynthesis of trehalose by *brevibacterium flavum*: Use of long range ^{13}C - ^{13}C coupling data to characterize triose phosphate isomerase activity. Bioscience Report (in press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 20015-02 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Estimation of Pollutant Concentrations in Groundwaters

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Warren T. Piver

Chemical Engineer

LMB

NIEHS

Other: F. Thomas Lindstrom

Associate Professor
Dept. MathematicsOregon State University
Corvallis, OR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Biochemical/Toxicology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.0

PROFESSIONAL

1.0

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A two-dimensional finite difference model was developed to examine the simultaneous transport of heat, moisture and chemicals in unconfirmed aquifers. The model allows for variable nodal spacing and greater flexibility in specifying soil properties and soil transport variables that are functions of position and moisture content. Numerical dispersion in the vicinity of steep gradients of moisture content, temperature and chemical concentration was minimized by using small increments in nodal spacing. The models were used to simulate transport in a wide variety of soil types providing a rapid method to analyze information about contamination of groundwater supplies from the use of pesticides and from the leachates migrating from buried waste chemical disposal sites.

Work was also completed on an examination of deterministic and stochastic models for contaminant transport in groundwaters. Both approaches have many procedural steps in common differing in the way variables that are functions of position and moisture content are approximated. User preference is a deciding factor for selecting one approach over another.

PROJECT DESCRIPTION

A. Estimation of Pollutant Concentration in Groundwaters Line Successive Over-relaxation (LSOR) methods were used to solve discretized finite difference representations of the three coupled field equations describing transport of chemicals in unconfined aquifers.

Experimental Approach and Scientific Justification: The one- and two-dimensional finite difference models were tested for a wide variety of soil and climatic conditions and were found to be valid representations for contaminant transport in unsaturated soils. Numerical dispersion in the solution was minimized by reducing nodal spacing size in the vicinity of steep gradients of moisture content, temperature and chemical concentration. One- and two-dimensional finite element models are currently being developed and tested. Simple chapeau functions are being used as the basis function set. A monograph is being prepared that describes these different models and provides examples of their application for modeling contaminant transport in unconfined aquifers.

B. Recent Accomplishments and Significance to Biomedical Research: These models make it possible to estimate exposure concentrations to chemicals in groundwaters from the use of pesticides and fertilizers and the migration of toxic chemicals from buried waste disposal sites.

C. Publications of past 18 months

Piver, W.T., and Lindstrom, F.T.: Waste disposal technologies for polychlorinated biphenyls. Environ. Health Persp. 59: 163-177, 1984.

Piver, W.T., and Lindstrom, F.T.: Simulating transport of organic chemicals in saturated/unsaturated soils. In Ziglio, G. (ed.). Ground Water Contamination with Organo-chlorine Compounds of Industrial Origin. Monduzzi Editore, Bologna, S.p.A., 1984, pp. 671-685.

Lindstrom, F.T., and Piver, W.T.: A Mathematical Model of the Vertical Transport and Fate of Toxic Chemicals in a Simple Aquifer. Technical Report No. 52, Corvallis, OR, Department of Mathematics, Oregon State University, April, 1985, 135 pp.

Lindstrom, F.T., and Piver, W.T.: Vertical transport and fate of low water solubility chemicals in unsaturated soils. J. Hydro. (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 30003-14 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Development of Analytical Methodology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Phillip W. Albro Research Chemist LMB NIEHS

Other: Carol E. Parker Chemist LMB NIEHS
Kun Chae Chemist LMB NIEHS

COOPERATING UNITS (if any)

EPA; Dow Chemical Company; Monsanto; New York Department of Health; University of Umea, Sweden; Health Prot. Br., Canada; National Fisheries Laboratory; Wright State University; University of Nebraska.

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0

PROFESSIONAL

0

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major effort this year has been concerned with development, validation, and application of methods for the accurate and reliable determination of chlorinated dibenzo-p-dioxins and dibenzofurans at low levels in environmental samples. Emphasis has been placed on accurate quantification and highly specific qualitative identification. Methods for extraction and cleanup of soil, adipose tissue, and liver have been developed and refined. A collaborative study has been started involving ten laboratories in three countries, to compare the analytical capabilities of these laboratories (which all use different techniques and approaches) for the low part-per-trillion level measurement of a series of dioxins and furans in human adipose tissue. The most reliable analytical approaches should be identifiable as a result of this study. Analysis of animal tissues and soil for 2,3,7,8-TCDD was used in another study to evaluate the bioavailability of TCDD bound to soil from Times Beach, Missouri.

A. Development of Analytical Methodology

Nature of Problem: Analytical data needed in support of biomedical research often exceeds present methodological capabilities. Development of adequate methodology is in itself a form of research, since the generation and interpretation of new types of data are essential to the process.

Objectives:

- A. General: To develop and refine methodology for the qualitative and quantitative determination of compounds and classes of compounds of general interest to the Institute and special interest to individual investigators.
- B. Current: (1) To develop reliable methodology for the extraction and cleanup of soil, adipose tissue, and liver for reproducible and accurate qualitative and quantitative determination of part-per-trillion levels of chlorinated dibenzo-p-dioxins and dibenzofurans; (2) to organize and perform a round-robin, blind study to evaluate a wide range of procedures used in various laboratories for the determination of part-per-trillion levels of chlorinated dibenzo-p-dioxins and dibenzofurans in human adipose tissue.

Approach and Justification: Extraction and cleanup procedures, involving relatively rapid, multi-sample capabilities, are based on elutriation techniques and optimized chromatographic procedures. Recoveries and precision are evaluated through the use of isotopically labeled compounds. Emphasis is placed on preparation of validly spiked surrogates, in which it can be demonstrated that exogenous (spiked) analyte equilibrates with endogenous analyte. The participants in the round-robin study, the dioxin and furan standards, and the human adipose tissue were recruited or acquired through the EPA. Preparation of blind, spiked tissue extracts to be sent to the participants for analysis required objective evidence that the 10 sets of 10 samples each were spiked uniformly and correspondingly, and was handled by NIEHS. Information on amounts of adipose needed and limits of determination were supplied by the participants. Breaking the blind code and evaluation of the analytical results is the responsibility of NIEHS. Participating laboratories were selected because they represent the complete range of analytical approaches to this determination, have all acquired international reputations for their previous work on dioxin/furan analysis, yet have never participated in a comparison of capabilities in a blind study where the right answers were known by independent means.

Recent Accomplishments and Significance: (1) The extraction techniques that have been developed give at least 99% recovery of dioxins and furans from liver. Recoveries from adipose tissue range from $97.5 \pm 3.6\%$ (S.D.) at the ppb level to $85.1 \pm 4.9\%$ (S.D.) at the low parts per trillion level. The recoveries from soil depend upon the amount of organic matter (humus) in the type of soil analyzed, but clay content does not seem to influence the results. Recoveries from 93-99% can be achieved at the parts-per-trillion level if the soil does not contain charcoal. Recovery through the cleanup procedures exceeds 90% in all cases, being greater for samples containing lower levels of total extractable organic matter. The cleanup procedure allows processing of approximately 20-24 samples per week, a significant increase over previous methods. Tetra- through octachlorinated dioxins and furans are recovered to equivalent extents, which is

not the case for most alternative methods. (2) Means for processing samples of adipose tissue 600 times larger than any we have used previously had to be developed for the round-robin study. We were able to successfully prepare uniformly spiked samples with picogram quantities of analytes at known levels, but to obtain uniformity it was necessary to spike after extraction. It seems to be beyond the state of the art to guarantee spiking uniformity if attempts are made to add the analytes at this level to unprocessed adipose tissue. Analytical results from the participating laboratories have not yet been received (3) 2,3,7,8-TCDD bound to soil from Times Beach, Missouri, and from the Minker-Stout site in Missouri, was bioavailable upon oral ingestion by rats and guinea pigs. Analysis of the livers of the test animals indicated that soil-bound TCDD was absorbed and accumulated between 30-50% as efficiently as TCDD in corn oil. TCDD bound to a low-humus, sandy soil was more efficiently "extracted" and bioaccumulated than TCDD bound to Times Beach soil, with TCDD bound to red clay intermediate.

The extreme acute toxicity of some of the chlorinated dibenzo-p-dioxins and dibenzofurans (e.g. LD₅₀ of 0.5 µg/Kg in the guinea pig for 2,3,7,8-TCDD and roughly 5 µg/Kg for 2,3,7,8-TCDF), combined with the present lack of understanding of the biochemical mechanism underlying the toxicity makes it necessary to perform biological experiments using extremely tiny amounts of material, often in the picogram and nanogram range. Dosing solutions involve amounts of material too small to weigh accurately, and determination of residual levels of test substances in tissues requires parts-per-trillion level analyses. Since these compounds are environmental pollutants, analytical results giving false negatives would contribute to a risk of poisoning, while false positives would have a major economic impact mandating extensive but unnecessary cleanup of the area sampled. In order to properly assess human health hazards from these compounds, among the fundamental data needed by various agencies are accurate and reliable measurements of the levels of dioxins and furans in the environment and in tissues of exposed subjects. Developing the means to obtain such data is a necessary precedent both to health hazard assessment and to well-controlled biomedical research. Further, both end-users must know how much confidence they can place in analytical data.

B. Plans for Subsequent Year

Since this project involves a response to expressed needs for development of methodologies, future work will depend upon what needs are expressed. There are no current plans for specific method development pending.

C. Publications of past 18 months

McConnell, E.E., Lucier, G.W., Rumbaugh, R.C., Albro, P.W., Harvan, D.J., Hass, J.R., and Harris, M.W.: Dioxin in soil: Bioavailability after ingestion of rats and guinea pigs. Science 223: 1077-1079, 1984.

Albro, P.W., Lorenzo, J., and Schroeder, J.L.: Simultaneous monitoring of 3-H and 14-C and determination of the 3-H/14-C ratio in the effluent of a liquid chromatograph using solid scintillators. Liquid Chromatography 2: 310-312, 1984.

Albro, P.W., Parker, C.E., Abusteit, E.O., Mester, T.C., Hass, J.R., Sheldon, Y.S., and Corbin, F.T.: Determination of the pKa values of Metribuzin and its metabolites: A comparison of spectrophotometric and potentiometric methods. J. Ag. Food Chem. 32: 212-217, 1984.

Tondeur, Y., Hass, J.R., Harvan, D.J., Albro, P.W., and McKinney, J.D.: Determination of suspected toxic impurities in Firemaster FF-1 and BP-6 by capillary impurities gas chromatography-high resolution mass spectrometry. J. Ag. Food Chem. 32: 406-410, 1984.

Tondeur, Y., Hass, J.R., Harvan, D.J., and Albro, P.W.: Computer-assisted determination of masses in high-resolution mass spectrometry by selected ion monitoring. Anal. Chem. 56: 373-376, 1984.

Tondeur, Y., Albro, P.W., Hass, J.R., Harvan, D.J., and Schroeder, J.: Matrix effect in determination of 2,3,7,8-tetrachlorodibenzo-p-dioxin by mass spectrometry. Anal. Chem. (in press).

Albro, P.W., Parker, C.E., Marbury, G.D., and Hernandez, O.: Spectrometric characterization of Metribuzin and its metabolites. Appl. Spectroscopy 38: 556, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 30066-09 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Theoretical Basis and Molecular Mechanisms of Biological Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: James D. McKinney Supervisory Research Chemist LMB NIEHS

OTHER: Urs Rickenbacher Visiting Fellow LMB NIEHS
Ricky Fannin Chemist LMB NIEHS
Sandy Jordan Biologist LMB NIEHS

COOPERATING UNITS (if any)

Toxicology Research and Testing Program, NTP, NIEHS
Duke Medical Center, Department of Radiology
Medical College of Wisconsin, Milwaukee

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Biochemical/Toxicology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The project has been incorporated into Z01 ES 50085-01 LMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 50046-07 LMB																
PERIOD COVERED October 1, 1984 to September 30, 1985																		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanisms of Chemically Induced Photosensitivity																		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: Colin F. Chignell</td> <td style="width: 30%;">Chief, LMB</td> <td style="width: 20%;">LMB</td> <td style="width: 20%;">NIEHS</td> </tr> <tr> <td>OTHER: Ann G. Motten</td> <td>Staff Fellow</td> <td>LMB</td> <td>NIEHS</td> </tr> <tr> <td>Robert D. Hall</td> <td>Staff Fellow</td> <td>LMB</td> <td>NIEHS</td> </tr> <tr> <td>Garry R. Buettner</td> <td>Expert</td> <td>LMB</td> <td>NIEHS</td> </tr> </table>			PI: Colin F. Chignell	Chief, LMB	LMB	NIEHS	OTHER: Ann G. Motten	Staff Fellow	LMB	NIEHS	Robert D. Hall	Staff Fellow	LMB	NIEHS	Garry R. Buettner	Expert	LMB	NIEHS
PI: Colin F. Chignell	Chief, LMB	LMB	NIEHS															
OTHER: Ann G. Motten	Staff Fellow	LMB	NIEHS															
Robert D. Hall	Staff Fellow	LMB	NIEHS															
Garry R. Buettner	Expert	LMB	NIEHS															
COOPERATING UNITS (if any) Enrico Gratton, Dept. of Physics, University of Illinois, Champaign, Urbana, IL.																		
LAB/BRANCH Laboratory of Molecular Biophysics																		
SECTION Molecular Biophysics																		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																		
TOTAL MAN-YEARS: <div style="text-align: center;">4.3</div>	PROFESSIONAL: <div style="text-align: center;">3.3</div>	OTHER: <div style="text-align: center;">1.0</div>																
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Light is known to interact with endogenous or exogenous chemical agents in the skin or eyes, to produce <u>photosensitization</u> (<u>phototoxicity</u> or <u>photoallergy</u>). While the initial step in all forms of photosensitivity must be the absorption of light by the chemical or its metabolites, the precise mechanisms of sensitization are unknown. The objective of this study is to determine whether light-induced free radicals or active oxygen species play a role in photosensitization. <u>Chlorpromazine</u> (CPZ) is an antipsychotic drug that causes both phototoxic and photoallergic reactions. UV irradiation of CPZ in aqueous solution resulted in the homolytic cleavage of the carbon-chlorine bond and the generation of an aryl radical which extracted a hydrogen atom from suitable donors. CPZ also photo-ionized when irradiated at 280 nm to give the CPZ cation radical. Thus it appears unlikely that the CPZ cation radical is involved in the phototoxicity of this drug. CPZ generated <u>singlet oxygen</u> (phosphorescence at 1270 nm) when irradiated in benzene, hexane and cyclohexane but not in aqueous or alcoholic solutions. The CPZ sulfoxide, which is formed in humans and other mammalian species, generated the hydroxyl radical and CPZ cation radical upon irradiation with near UV light. <u>Hematoporphyrin derivative</u> (HPD) and light convert oxygen to hydroxyl free radical and hydrogen peroxide in the presence of light. HPD and light also initiated an oxygen-dependent formation of thiyl radical from cysteine. <u>Halogenated salicylanilides</u> eg. 3,3',4',5-tetrachlorosalicylanilide (TCSA), 3,4',5-tribromosalicylanilide (TBSA) and N-butyl-4-chlorosalicylanilide (buclosamide) all generated aryl radicals during photodehalogenation. Irradiation of TBSA with glutathione or cysteine resulted in the generation of the corresponding thiyl radicals. Thus it appears possible that the skin photoallergy of these compounds may result from the reaction of these radicals with proteins to form antigens. </p>																		

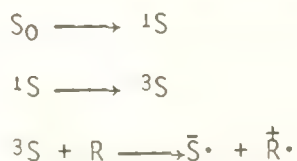
PROJECT DESCRIPTION

A. Mechanisms of Chemically Induced Photosensitivity

Nature of Problem: Light is known to interact with chemical agents in exposed tissues, such as the skin or eyes, to produce photosensitization. The chemical agent may be endogenous (protoporphyrin in erythropoietic protoporphyria), a drug (sulfonamides, declomycin, chlorpromazine), a topical agent (4-aminobenzoic acid and its esters in sunscreens; halogenated salicylanilides in soaps) or an environmental agent (polycyclic aromatic hydrocarbons in coal tar; amyl esters of 2-aminobenzoic acid in printer's ink). The photosensitivity response may be one of two types, phototoxic or photoallergic. The phototoxic reaction generally occurs during a subject's first exposure to sunlight, after the administration or topical application of a chemical or drug, and usually takes the form of an exaggerated erythematous response ("sunburn"). Photoallergic individuals may also exhibit an initial erythematous reaction. As this subsides, delayed abnormal responses may begin to appear including papular, eczematous and urticarial reactions. Such reactions to light may persist for months after avoidance of the photoallergen.

While the initial step in all forms of photosensitivity must be the absorption of light by the chemical or its metabolites, the precise mechanisms of phototoxicity and photoallergy are unknown. The close similarity between phototoxicity and the normal erythematous response to excessive sunlight exposure suggests that the mechanisms are the same or very similar. In photoallergy, the agent is converted to a chemically reactive species (hapten) which then combines with a protein or other macromolecule (carrier) to form an allergen. The mechanism of the subsequent immunologic response is presumably similar to other types of delayed hypersensitivity.

When the ground state (S_0) of a molecule absorbs a photon of light it is converted to a very short-lived singlet excited state (1S) which rapidly goes to a long-lived excited triplet state (3S). Most photosensitized reactions in biological systems are mediated by the triplet state of the sensitizer. Subsequent reactions can proceed by a number of different pathways, depending on the chemical nature of the sensitizer and the substrate as well as the reaction conditions. Two major types of processes may occur, termed Type I and Type II. In Type I (free radical or redox reactions) reactions the triplet sensitizer molecule may abstract an electron (or hydrogen atom) from the substrate molecule (R) to give a semi-reduced (free radical) form of the sensitizer (S^\cdot) and a semi-oxidized (free radical) form of the substrate (R^\cdot). These processes may be summarized as follows:



The semi-reduced form of the sensitizer may also react directly with oxygen to

give the superoxide radical ($\text{O}_2^{\cdot-}$)



In Type II (energy transfer) reactions, electronic excitation energy is transferred from the triplet state sensitizer to ground state oxygen to give a highly electrophilic excited singlet state ($^1\text{O}_2$) of oxygen.



This form of oxygen reacts with many kinds of biomolecules much more rapidly than ground state oxygen.

Objectives: The objectives of this research are to determine the precise mechanisms whereby chemical agents produce photosensitization. This is achieved by first identifying those reactive species (free radicals, superoxide anion, singlet oxygen) that are generated when the photosensitizer is irradiated with light. This is then followed by a detailed study of the chemistry of the reactive species under conditions that are as close to physiological as possible. Finally, direct evidence is sought for the involvement of the reactive species that have been identified in the known biological response to a given photosensitizer. The biological studies are initially carried out with simple systems such as liposomes or the human erythrocyte. This work is then extended to more complex systems such as cells grown in culture or isolated from skin.

Experimental Approach: Free radicals may be detected, identified and quantitated by electron spin resonance (ESR) spectroscopy. However, since most free radicals generated photolytically are chemically reactive they cannot always be observed by direct ESR. For such radicals the technique of spin trapping may be employed. This procedure uses diamagnetic organic molecules (spin traps) that react with free radicals to produce stable nitroxide radicals (spin adducts). The identity of the parent radical may often be determined from the ESR spectrum of the resultant spin adduct.

Singlet oxygen may also be detected by ESR. One technique involves the use of a hindered amine (2,2,6,6-tetramethylpiperidine) which upon reaction with singlet oxygen yields a stable nitroxide free radical (2,2,6,6-tetramethylpiperidine-1-oxyl). A second procedure involves the measurement of oxygen consumption by monitoring the effect on the linewidth of an exogenously added nitroxide. In both cases the presence of singlet oxygen is confirmed by the use of specific quenchers such as dibenzofuran, dimethylfuran and DABCO. Singlet oxygen may also be detected directly by observing its luminescence at 1270 nm.

Recent Accomplishments

Chlorpromazine: Chlorpromazine [2-chloro-N-(3-dimethylaminopropyl)phenothiazine] is a frequently prescribed antipsychotic drug which causes both phototoxic and photoallergic reactions. Irradiation of chlorpromazine is known to produce the chlorpromazine cation radical, as well as other photoproducts which may go through radical intermediates. As a preliminary to studying the radical photoproducts of chlorpromazine, the high-resolution ESR spectra of the chemically generated aqueous cation radicals of six phenothiazine derivatives were obtained and fully analyzed.

Under UV irradiation the radical resulting from dechlorination of chlorpromazine was trapped using 2-methyl-2-nitrosopropane (MNP) to provide the first proof that the well-known dechlorination does in fact proceed by homolytic cleavage of the carbon-chlorine bond. The reactivity of the dechlorination product is similar to that of the phenyl radical as shown by its ability to extract hydrogen atoms from donors. Our results suggest that the dechlorination product is sufficiently reactive to account for the observation that chlorpromazine is more phototoxic than its parent promazine. In the presence of oxygen both chlorpromazine and promazine form a superoxide-dismutase-insensitive oxygen-centered intermediate which, when trapped by 2,2-dimethyl-1-pyrroline-N-oxide (DMPO), rapidly decays to DMPO-OOH and subsequently to DMPO-OH. Chlorpromazine readily undergoes photoelectron ejection only when it is excited into the second excited singlet state ($\lambda < 280$ nm). This previously unknown wavelength dependence of photoionization does not occur in the parent promazine. We have shown by laser flash photolysis (courtesy of the Center for Fast Kinetic Research, Austin, Texas) that the often-observed photoionization which takes place when chlorpromazine is excited into the lowest excited singlet state ($\lambda > 310$ nm) is a biphotonic process involving the triplet state, contrary to previous reports. This suggests that formation of the cation radical via photoionization is not important in photosensitization by sunlight.

A spin-trapping investigation of the metabolite chlorpromazine sulfoxide, formed in humans and several other mammalian species, shows that the sulfoxide, when irradiated with near UV light, produces large amounts of the highly reactive hydroxyl radical ($\cdot\text{OH}$) as well as the cation radical. To further examine the role of the sulfoxides, measurements of their fluorescence and phosphorescence spectra and lifetimes were carried out. Although the UV absorption spectra of chlorpromazine and its sulfoxide overlap, their fluorescence spectra are distinctive and well separated. By monitoring the fluorescence, we were able to follow the conversion of chlorpromazine to its sulfoxide in air-saturated aqueous solution, as well as the photolysis of sulfoxide back to chlorpromazine in the simultaneous absence of O_2 and presence of ascorbate (vitamin C). Animal species which do not form the sulfoxide metabolically do not develop ocular complications, and thus it has been suggested that the sulfoxide may be responsible for ocular photosensitivity. Efforts to delineate more clearly the photochemistry of the sulfoxide using ESR, fluorescence spectroscopy, and conventional flash photolysis are continuing.

Many photosensitizers react with molecular oxygen to give singlet oxygen, a powerful oxidizing agent. Detection of the emission of singlet oxygen produced by photoexcited chlorpromazine shows that this type of electronic energy transfer occurs in benzene, hexane, and cyclohexane, but is below present limits of detection in aqueous solution and in methanol. Thus, Type II photosensitization in vivo may occur only when chlorpromazine is present in nonaqueous locations such as cell membranes.

Further studies of chlorpromazine photolysis products, including the Forrest free radical metabolite, will use flash photolysis, fluorescence, and ESR spectroscopy in an effort to analyze the entire photolysis reaction scheme of the phenothiazines.

Anthracene: Anthracene, a component of coal tar, is a potent photosensitizing agent both in vivo and in vitro. Studies by others in mice have shown that anthracene is photocarcinogenic. Irradiation of anthracene in ethanol produced singlet oxygen as detected by the hindered amine technique and singlet oxygen phosphorescence at 1270 nm. The rate of singlet oxygen production decreased in the presence of sodium azide and was enhanced when deuterioethanol was employed. Other studies have suggested that superoxide is also formed.

Hematoporphyrin derivative: Photodynamic therapy with hematoporphyrin derivative (HPD) is currently being developed as a treatment for malignant skin disease. There is clear evidence that the cytotoxic agent is derived from oxygen. Cytotoxicity has been attributed to singlet oxygen, an excited state of molecular oxygen, and to free radicals. We have demonstrated that hematoporphyrin derivative and light are able to convert oxygen to hydrogen peroxide and the hydroxyl free radical in the presence of ascorbate. This may be of importance in the eye as the aqueous humor has a particularly high level of ascorbate (1 mM). In addition, HPD and light appeared to initiate an oxygen-dependent formation of thiyl radical from cysteine as seen by spin trapping. While the mechanism of this process is not yet understood, it appears that singlet oxygen reacts with the ionized form of cysteine. This product then forms the radicals observed. These observations suggest that cellular thiols may well be a prime target in HPD photosensitization.

Halogenated salicylanilides: Several halogenated salicylanilides eg. 3,3',4',5-tetrachlorosalicylanilide (TCSA), 3,4',5-tribromosalicylanilide (TBSA) and N-butyl-4-chlorosalicylanilide (Buclosamide) are known to cause contact photoallergy in human and animal subjects. Irradiation ($\lambda > 300$ nm) of TCSA in aqueous alcoholic solutions resulted in the sequential loss of the 3,5 and 4'-chloroatoms to yield 3'-chlorosalicylanilide. Under the same conditions TBSA photo-eliminated the 3,5 and 4'-bromoatoms and buclosamide lost a single chlorine atom. Spin trapping studies with MNP in alkaline aqueous solution provided evidence for the photogeneration of phenyl radicals from all three compounds. Irradiation of TBSA with glutathione or cysteine resulted in hydrogen abstraction from the sulfhydryl group and the generation of the corresponding thiyl radical (RS \cdot). When TCSA was irradiated in 50% ethanol a carbon-centered radical derived from the solvent (CH₃CHOH) was detected. Similar results were obtained with TBSA and buclosamide. These findings indicate that TCSA, TBSA and buclosamide all undergo photodehalogenation to give the corresponding highly reactive phenyl radicals. Thus it appears possible that the photoallergy of these compounds may result from a reaction of these radicals with proteins and other biological macromolecules to form antigens.

Amiodarone: Amiodarone [2-butyl-3-(3',5'-diiodo-4'- α -dimethylaminoethoxybenzoyl)-benzofuran] is an antiarrhythmic drug that often produces cutaneous phototoxicity. In addition to photosensitivity, which is characterized by intense burning, erythema and swelling of exposed areas, many patients also develop a rare slate-grey pigmentation. The formation of the pigment has been attributed to the deposition of lipofuscin (not melanin) in exposed skin. We have found that irradiation ($\lambda > 300$ nm) of amiodarone in aqueous ethanol results in the

rapid loss of a single iodine atom. Spin trapping studies in aqueous solution with MNP have shown that upon irradiation amiodarone generates a highly reactive aryl radical. When linoleic acid was present the drug-derived radical was replaced by a carbon-centered linoleyl radical. If such a reaction occurred in vivo the lipid radicals would react with oxygen to produce the corresponding peroxy radicals. Thus it appears possible that lipofuscin deposited in the skin of patients receiving amiodarone may result from lipid peroxidation initiated by the drug in the presence of light.

Instrument Development: Many environmental agents, eg. polycyclic aromatic hydrocarbons, have interesting fluorescence properties which can be used to monitor their interaction with biological molecules. Work has therefore continued to improve the utility of available fluorometers. A simple procedure has been devised to adjust phase modulation spectrometers so that reproducible lifetime measurements can be determined. Studies with reference compounds for lifetime studies have continued. Initial results have shown that the greatest accuracy in lifetime determinations can be achieved using reference compounds with lifetimes longer than the sample lifetimes.

Although it has been suggested that singlet oxygen may play an important role in many pathological biochemical reactions, direct spectroscopic evidence for its existence has been lacking until recently. However, direct observation of the near-infrared (1270 nm) luminescence of singlet oxygen has made it possible to study its chemistry without chemical perturbation of a biological system. A highly sensitive singlet-oxygen detection system utilizing a liquid nitrogen-cooled germanium diode has been constructed. The responsivity of the instrument has been documented and studies of simple homogeneous and heterogeneous (micellar) systems have been carried out in order to determine the efficiency of singlet oxygen production by several different categories of photosensitizers: polycyclic aromatic hydrocarbons, benzoxazole and phenothiazine derivatives, and hematoporphyrin derivative. The effect of different solvents and biological quenching agents like cysteine and ascorbic acid on singlet oxygen emission is currently under investigation.

The flash photolysis approach is particularly useful for examining decay from the triplet state, which commonly occurs in the microsecond region. Furthermore radicals, which often have decay times on the order of milliseconds or seconds, can also be detected by flash photolysis. We have been unable to detect the primary radicals formed during the photolysis of a number of skin photosensitizing chemicals using either conventional ESR or spin trapping. It seems reasonable to assume that such radicals, if generated, have lifetimes that are too short. The flash photolysis technique offers an additional approach to the detection and identification of such transient species. A conventional flash photolysis system has been purchased and is now operational. We have interfaced the system to an Apple IIe computer so the appropriate data analysis can be performed.

The hyperfine splitting constants obtained from an ESR spectrum contain detailed structural and mechanistic information. However, the analysis of an ESR spectrum by eye using trial-and-error is often a time-consuming process and may not always be successful. A computer-aided correlation approach to solving ESR

spectra has been recently developed and implemented on a mainframe computer by other workers. We have modified the correlation analysis for use on a micro-computer interfaced to the ESR spectrometer and developed much faster algorithms for the correlation procedures in order to make using the small computer feasible. Matching a computer-simulated spectrum with an experimental spectrum can now be done automatically using our programs if the approximate hyperfine splittings are known; in favorable cases the hyperfine splittings can be extracted from the experimental spectrum as well. The correlation approach works successfully for well-resolved spectra even if they are noisy, but is only moderately helpful for poorly resolved spectra. Efforts are under way to develop an alternative approach involving separating spectra into component parts, which looks promising for poorly resolved spectra with low noise.

B. Plans for Subsequent Year

Photolysis of skin photosensitizers

Studies will continue on the photolysis of various skin photosensitizing agents including the salicylanilides, tetracyclines, nalidixic acid, bithionol, fentichlor, amiodarone and N-butyl-4-chlorosalicylamide. Additional work is also planned on a series of polycyclic aromatic hydrocarbons eg. anthracene, fluoranthene, pyrene, phenanthrene, chrysene, carbazole, acridine and phenanthridine all of which are known components of coal tar.

Instrumental Development

Work is planned in the development of three spectroscopic procedures to aid in the photolysis studies.

Singlet oxygen detector When studies on model systems which generate singlet oxygen have been completed more complex systems including erythrocyte ghost membranes and isolated cells will be examined. Singlet oxygen has been implicated in the mechanism of a number of enzymes including xanthine oxidase, microsomal lipid oxidase, prostaglandin synthase, and horseradish peroxidase. Much of the evidence for singlet oxygen involvement has been based on the emission of chemiluminescence or the effects of inhibitors. With the availability of a system that permits the direct detection of singlet oxygen, the possible role of this active oxygen species in oxidative biological processes can be determined unequivocally.

Computer solution of ESR spectra: A high-speed (12 MHz) processor will be slaved to the ESR-interfaced computer and machine-language coding will be used to speed up data processing. Our goal is to develop a program fast enough to simulate and solve spectra interactively. The potential of the two analysis approaches now being used will be further explored.

Photoacoustic spectroscopy: Photoacoustic spectroscopy is a technique whereby the optical absorption spectra of opaque samples, such as solids, surfaces, gels and intact biological samples, may be obtained. In addition this technique can provide information on thermal diffusivity, depth profile of absorbing species, calorimetric properties, excited state lifetime, emission quantum yields, and the action spectrum of photoreactions. Photoacoustic spectroscopy should be ideal

for studying the interaction of photosensitizing agents with the skin. A photoacoustic spectrometer has been modified for such studies and interfaced to an Apple IIe computer. Initial work has indicated that the spectra of biological samples such as bovine serum albumin can be recorded with this system. Other work with colored dyes adsorbed onto cellophane has shown that it is possible to record absorption spectra at different levels in a multilayered system. This procedure will now be employed to study the fate of photosensitizing chemicals on skin taken from "hairless" mice. If this is successful an attempt will be made to design a cell that can be used in in vivo studies. It should also be possible, using this technique, to monitor the diffusion rate of chemicals and drugs across the skin.

C. Publications of past 18 months

Fischer, V., Harrelson, W.G., Chignell, C.F., and Mason, R.P.: Spectroscopic studies of cutaneous photosensitizing agents. V. Spin-trapping and direct electron spin resonance investigations of the photoreduction of gentian (crystal) violet. Photochem. Photobiophys. 7: 111-119, 1984.

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Chignell, C.F.: Use of spin labels as enzyme probes. In Holtzman, J.L. (Ed.): Spin Labeling in Pharmacology. New York, Academic Press, 131-157, 1984.

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Motten, A.G. and Chignell, C.F.: Electron spin resonance of cation radicals of phenothiazine derivatives. Org. Magnet. Resonance, in press.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 50077-03 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Free Radical Intermediates of Antiparasitic Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Robert Docampo Visiting Scientist LMB NIEHS

OTHER: Silvia N.J. Moreno Visiting Fellow LMB NIEHS
Ronald P. Mason Research Chemist LMB NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Molecular Biophysics

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.1

PROFESSIONAL

1.1

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recently it has become increasingly apparent that many reactive intermediates of xenobiotics are free radicals. The formation of free radicals, including oxygen-derived radicals, may lead to extensive cellular damage. The consequences of radical-initiated reactions may be the immediate death of the cells or may be more subtle and delayed as evidenced by the development of neoplasms. In a study of *Trypanosoma cruzi* (the agent of Chagas' disease), crystal violet, a triarylmethane dye widely used by blood banks to eliminate transmission of Chagas' disease by transfusion, was found to undergo a one-electron reduction to produce a carbon-centered free radical. Both radical formation and the trypanocidal action of crystal violet, were enhanced by light. The photodynamic action of gentian violet in blood was apparently mediated by ascorbic acid oxidation. In addition, we demonstrated that photolysis of gentian violet in aqueous medium saturated with O₂ does not generate singlet oxygen. The photodynamic action of rose bengal on *T. cruzi* has also been established. These observations may have important chemoprophylactic implications, since illumination of blood to be transfused may result in a better therapeutic ratio. We provided evidence for the one-electron reduction of benznidazole by *T. cruzi* homogenates and described differences between nifurtimox and benznidazole modes of action on *T. cruzi*. In addition, we provided evidence for the involvement of O₂ radicals in the killing of *T. cruzi* by polymorphonuclear leukocytes. Finally the ability of *Tritrichomonas foetus* (the agent of trichomoniasis in cattle) hydrogenosomal and cytosolic fractions to generate nitroimidazole and nitrofuran anion radicals was established. These results support the role of air oxidation as a detoxification reaction for the nitroimidazoles anion radicals and the involvement of a ferredoxin in their formation. On the other hand, redox cycling of nitrofurans, with formation of high steady state concentrations of oxygen-derived radicals, might be of toxicological significance.

A. Free Radical Intermediates of Antiparasitic Drugs

Nature of Problem: Parasitic diseases are the most widespread of all the major human diseases, currently affecting about three billion people. Effective drug treatment is nonexistent for many of them, and most of the available drugs, when adequately tested, have been shown to have mutagenic, cytotoxic, and carcinogenic activities. In recent years it becomes increasingly apparent that many reactive intermediates of xenobiotics are free radicals. The formation of free radicals, including oxygen-derived radicals, may lead to extensive cellular damage. The consequences of radical-initiated reactions may be the immediate death of the cells or may be more subtle and delayed as evidenced by the development of neoplasms.

Objectives: The aim of this investigation is (a) to identify free radical intermediates generated by antiparasitic drugs and phagocytic cells in the presence of parasites by electron spin resonance spectroscopy, and (b) to investigate the ability of parasite and mammalian cells and their subcellular fractions to generate free radicals from antiparasitic compounds.

Experimental Approach and Scientific Justification: The project will be accomplished by (a) Isolation of parasite and mammalian cells and their fractions using known methods improved when necessary. (b) Determination of free radical intermediates from antiparasitic compounds by direct ESR spectroscopy or by using spin-trapping agents. (c) Study of the enzymes and cofactors involved. (d) Computer simulation of the spectra obtained to characterize radicals.

The information obtained in these studies will help make future drug development possible on a more rational basis than has been possible hitherto. In addition, the study of the mode of action of existing chemotherapeutic and chemoprophylactic agents is necessary to maximize efficacy and to minimize toxicity.

Recent Accomplishments and Significance

1. Pathways involved in free radical intermediates generation by crystal violet

In our previous studies with intact trypanosomes, crystal violet was found to undergo a one-electron reduction to produce a carbon-centered free radical as demonstrated by electron spin resonance spectroscopy. Because the formation of this free radical and the trypanocidal action of crystal violet were enhanced by light, we postulated that by illumination of the infected blood it would be possible to decrease the optimal trypanocidal concentration of crystal violet, avoiding possible side effects of drug. Furthermore, the addition of reducing agents might enhance the effect of light on the trypanocidal action of crystal violet. In preliminary experiments we detected a photodynamic effect of crystal violet against T. cruzi trypomastigotes in blood. This effect was apparently mediated by ascorbic acid oxidation since (a) illumination of blood containing crystal violet with visible light resulted in the detection of ascorbyl radical; continued illumination resulted in a disappearance of the signal, (b) similar results were observed after illumination of a solution containing crystal violet and ascorbic acid in buffer; (c) illumination of buffer containing crystal violet and ascorbic acid resulted in an enhanced formation of OH^\bullet radical and H_2O_2 ; and (d) illumination of whole blood containing T. cruzi trypomastigotes

resulted in an enhanced killing of the parasites, and this effect was further enhanced by the addition of ascorbic acid to the blood. In addition, we demonstrated that photolysis of crystal violet in aqueous medium saturated with O_2 does not generate singlet oxygen.

2. Pathways involved in free radical intermediates generation by nitrocompounds
Previous attempts to demonstrate benznidazole reduction to a nitro anion radical in the presence of T. cruzi intact cells, and mitochondrial and microsomal fractions under conditions similar to those used with the nitrofuran nifurtimox were unsuccessful, and it had been speculated that this reduction might not involve a one-electron transfer to this nitrocompound. During this period we provided evidence of one-electron reduction of benznidazole by T. cruzi homogenates and described differences between nifurtimox and benznidazole modes of action on T. cruzi.

3. Free radical formation by other trypanocidal agents Because light enhances the trypanocidal action of crystal violet, the role of light in the trypanocidal action of dyes has become of interest. In addition, since dyes absorbing light are among the few natural systems that permit us to study the effects of singlet oxygen production, we studied the effects of rose bengal, a known singlet oxygen producer, on T. cruzi structure and viability. Rose bengal in the presence of light and oxygen mounted an oxidative attack on T. cruzi. The production of lipid hydroperoxides was demonstrated by thin layer chromatography and severe ultrastructural alterations compatible with an increased permeability of the cells which led to gradual osmotic swelling and ultimately to lysis were observed by electron microscopy. As a result of this treatment the infectivity of T. cruzi trypomastigotes in mice was abolished. In addition, under anaerobic conditions, rose bengal was found to undergo a one-electron reduction in intact T. cruzi epimastigotes to produce a carbon-centered free radical as demonstrated by ESR spectroscopy. The formation of this radical was also enhanced by light.

4. Free radical formation by phagocytic cells in the presence of T. cruzi The temperature-dependence of some processes involved in the killing of sensitized T. cruzi epimastigotes by human polymorphonuclear leukocytes (PMN) was determined. The rate of the reactions was related to the temperature of incubation according to the Arrhenius equation and the apparent energies of activation (E_a) were calculated. The E_a values separated these complex reactions into two groups: one with E_a of about 10 kcal/mol for the phagocytosis of the parasites and the release of lysosomal enzymes by PMN, and the other with E_a of about 22 kcal/mol for the cytotoxicity against sensitized T. cruzi, the rate of oxygen consumption by PMN, and the lysis of the parasites by added hydrogen peroxide.

5. Redox cycling of nitrocompounds as a detoxification reaction (nitroimidazoles) or as a toxic mechanism (nitrofurans) in trichomonads The anaerobic incubation of metronidazole with intact Trichomonas foetus cells in the presence of glucose results in the appearance of a characteristic ESR spectrum corresponding to the nitro-anion radical. The signal is also observed in the absence of added glucose, indicating that endogenous reducing substrates can be used by the cells for metronidazole reduction. However, the steady-state concentration of the nitro anion radical is 40-50% higher with added glucose.

The ability of intact *T. foetus* to reduce nitro-compounds is not limited to metronidazole. Incubation of several nitrofurans (nifuroxine, nifurtirom, nitrofurantoin, nitrofurazone), 2-nitroimidazoles (benznidazole, misonidazole), and 5-nitroimidazoles (ronidazole, secnidazole, MK-436, fexinidazole, ornidazole) with intact *T. foetus* also generates multiline ESR spectra corresponding to their respective nitro anion radicals. The anaerobic incubation of metronidazole and other nitrocompounds with the *T. foetus* hydrogenosomal fraction in the presence of pyruvate and CoA also generates the corresponding nitro anion radicals. The addition of purified ferredoxins causes a marked stimulation of the reduction of metronidazole and other 5-nitroimidazoles to their anion radicals, suggesting a role of a ferredoxin in this process. According to the proposed redox cycling of nitrocompounds; aerobic incubations of *T. foetus* hydrogenosomal fraction with metronidazole result in the consumption of O_2 and the generation of $O_2^{\cdot -}$ and H_2O_2 . The addition of purified ferredoxins also causes a marked stimulation of metronidazole-induced O_2 consumption by these preparations. A very high concentration of metronidazole is necessary for the detection of an increase in the O_2 consumption and $O_2^{\cdot -}$ and H_2O_2 formation by the hydrogenosomal fraction. In addition, metronidazole cannot stimulate O_2 consumption in intact cells when used at low concentrations (less than 6 mM). In contrast with the results obtained with metronidazole, lower concentrations of nitrofurans are necessary for the detection of an increase in the O_2 consumption and $O_2^{\cdot -}$ and H_2O_2 formation by the hydrogenosomal fraction, and this process is not stimulated by ferredoxins. In addition, nitrofurans stimulate O_2 consumption in intact cells when used at low concentrations (less than 6 mM). In conclusion, redox cycling of metronidazole under aerobic conditions might be considered as a detoxification reaction by inhibiting net reduction of the drug, thereby inhibiting its uptake. The resulting low steady-state concentrations of $O_2^{\cdot -}$ are easily detoxified by the superoxide dismutase present in these parasites. On the other hand, redox cycling of nitrofurans or other compounds with more positive reduction potentials will result in formation of high steady-state concentrations of oxygen-derived metabolites that might be of toxicological significance.

6. The literature concerning free radical formation by antiparasitic drugs and phagocytic cells and the biochemical toxicology of drugs used in the chemotherapy and chemoprophylaxis of Chagas' disease and Trichomoniasis was reviewed.

B. Plans for subsequent year

The chemoprophylactic potential of the photodynamic action of gentian violet will be explored. Since photodynamic therapy usually results in immediate observable damage to cells, irradiation of the blood to be transfused would be very convenient in emergency situations. On the other hand, because light also enhances the genotoxicity of gentian violet the role of light in the possible toxicological effects of gentian violet has become of interest.

We propose to explore the optimal conditions to obtain a photodynamic action of gentian violet on T. cruzi trypomastigotes in blood, i.e., wavelength, light intensity, dye concentration, parasite number, presence of reducing agents known to enhance gentian violet free-radical generation (ascorbic acid, cysteine, glutathione, NADH, NADPH). On the other hand, the effect of these treatments on the blood will be examined, i.e., hematocrit, free plasma hemoglobin, plasma Na^+ and K^+ , erythrocyte ATP levels, erythrocyte 2,3-diphosphoglycerate levels, hemoglobin abnormalities, and morphological alterations.

The formation of a carbon-centered metabolite of gentian violet by the action of rat liver microsomes and a NADPH-generating system under anaerobic conditions has been described. Apparently, this free radical is formed in liver (at least in part) by a one-electron transfer from reduced cytochrome P-450, as indicated by its CO and metyrapone sensitivity. The same carbon-centered radical of gentian violet was identified in anaerobic incubations of intact T. cruzi epimastigotes or trypomastigotes, or T. cruzi epimastigote homogenate in the presence of NADH or NADPH. Furthermore, cellular suspensions of microflora from either human, rat or chicken feces, as well as pure cultures of 9 genera of strict and facultative anaerobes reduced gentian violet to leucogentian violet. Apparently, the carbon-centered radical is an intermediate in the anaerobic reduction of gentian violet to leucogentian violet. However, neither the formation of leucogentian violet under anaerobic condition nor the formation of superoxide anion under aerobic conditions by rat liver microsomes or T. cruzi cells or subcellular fractions was investigated. We propose to study these reactions. Leucogentian violet will be identified by HPLC. Superoxide anion formation will be detected by biochemical assays or by spin-trapping techniques.

In addition to the reductive pathway, an oxidative metabolism of gentian violet has been described. Gentian violet is demethylated by liver microsomes from different animals supplemented with an NADPH-generating system. The major metabolites isolated by solvent extraction and HPLC were identified as pentamethylpararosaniline and the isomeric N,N,N',N''-tetramethylpararosaniline a commonly observed phenomenon involving hemoproteins of the mixed-function oxidase system. A recent example is the demethylation of the antitumor agent, hexamethylmelamine, by mouse liver microsomes. The vast majority of cases investigated so far, however, involve neutral molecules. Gentian violet differs in that it is a resonance-stabilized cation at physiological pH. Therefore, we propose to identify the enzyme responsible for the N-demethylation reaction of gentian violet in liver microsomes. The N-demethylase activity of liver microsomes will be assayed by measuring the amount of formaldehyde formed. The effect of inhibitors of cytochrome P-450 (CO, metyrapone, SKF-525-A) and of NADPH cytochrome c reductase (NADP⁺, p-hydroxymercuribenzoate) will be assayed. The N-demethylase reaction will also be assayed with microsomes obtained from rats induced with phenobarbital or 3-methylcholanthrene. Since a monooxygenase system has been described in T. cruzi epimastigotes, the N-demethylation of gentian violet by T. cruzi microsomal fractions will also be investigated.

Finally, N-demethylation reactions can also be catalyzed by peroxidases such as horseradish peroxidase (HRP) and prostaglandin synthetase (PGS); the peroxidase

mediated reactions involve the formation of free radical intermediates as indicated by ESR spectroscopy. Moreover, cytochrome P-450 can also function as a peroxidase in the presence of organic hydroperoxides or H_2O_2 , and this hemoprotein has been found capable of catalyzing a variety of reactions, including N-demethylations by a peroxidative mechanism. The peroxidative cooxidation pathway, found in tissues rich in prostaglandin endoperoxide synthetase is ubiquitous throughout the body. The PGS system is now being studied as an alternate activating pathway in xenobiotic metabolism. Gentian violet has aromatic rings that are N,N-dimethyl substituted and closely resembles compounds that have been shown to be good substrates for N-dealkylation by PGS. The basic structure requirement for N-dealkylation by PGS is an aromatic ring system that is N-alkyl or N,N-dialkylsubstituted. Therefore, it seems likely that this dye may be N-dealkylated in a similar manner. Taking into account the carcinogenic potential of the resultant derivatives, this pathway of metabolic activation of gentian violet will be investigated.

C. Publications of past 18 months:

Moreno, S.N.J., Mason, R.P., and Docampo, R.: Distinct reduction of nitrofurans and metronidazole to free radical metabolites by *Tritrichomonas foetus* hydrogenosomal and cytosolic enzymes. J. Biol. Chem., 259: 8252-8259, 1984.

Cruz, F.S., Lopes, L.A.V., DeSouza, W., Moreno, S.N.J., Mason, R.P., and Docampo, R.: The photodynamic action of rose bengal on *Trypanosoma cruzi*. Acta Trop. 41: 99-108, 1984.

Docampo, R., and Moreno, S.N.J.: Free Radical Intermediates in the Antiparasitic Action of Drugs and Phagocytic Cells. In Pryor, W.A. (Ed.): Free Radicals in Biology, Vol. 6, New York, Academic Press, 1984, pp. 243-288.

Docampo, R., and Moreno, S.N.J.: Free radical metabolites in the mode of action of chemotherapeutic agents and phagocytic cells on *Trypanosoma cruzi*. Rev. Infect. Dis. 6: 223-238, 1984.

Docampo, R., and Moreno, S.N.J.: Biochemical Toxicology of Antiparasitic Drugs Used in the Chemotherapy and Chemoprophylaxis of American Trypanosomiasis (Chagas' disease). In Hodgson, E., Bend, J.R. and Philpot, R.M. (Eds.): Reviews in Biochemical Toxicology. New York, Elsevier Biomedical, in press.

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Cardoni, R.L., de Titto, E.H., and Docampo, R.: On the mechanism of killing of *Trypanosoma cruzi* by human polymorphonuclear leukocytes. Experientia 41: 89-91, 1985.

Moreno, S.N.J. and Docampo, R.: The mechanism of toxicity of nitro compounds used in the chemotherapy of trichomoniasis. Environm. Hlth. Perspect., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 50078-03 LMB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Radical Anion Metabolites		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	Ronald P. Mason	Research Chemist LMB NIEHS
OTHER:	Kim Morehouse	Staff Fellow LMB NIEHS
COOPERATING UNITS (if any) Clinical Pharmacology, VA Hospital, Minneapolis, MN Department of Pharmacology, UNC, Chapel Hill, NC		
LAB/BRANCH Laboratory of Molecular Biophysics		
SECTION Molecular Biophysics		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The objective of this study is to determine the role played by free radicals in the <u>reductive metabolism</u> of xenobiotics. The anaerobic incubation of almost all <u>nitroaromatic xenobiotics</u>, e.g., nitrobenzene, by the microsomal, mitochondrial, or cytosolic fractions of rat liver in the presence of either NADH or NADPH, leads to a multiple-line electron spin resonance spectrum characteristic of the nitro anion free radical. We have now demonstrated nitro anion radical formation by mitochondria using endogenous cofactors. Nitro drugs do not affect mitochondrial respiration, in particular the coupling to ADP. The sites of nitro reduction, as determined by inhibitors of the mitochondrial transport chain, appear to be NADH dehydrogenase and outer-membrane NAD(P)H cytochrome c reductase.</p> <p>Halogen-substituted nitro compounds are radiosensitizers and are among the most toxic nitro compounds. Loss of halide by the nitroaromatic anion forms a very reactive carbon-centered free radical, detected by spin trapping, which reacts with cellular macromolecules. The irreversible binding of these nitro compounds to DNA, protein, etc. may be inhibited by spin traps.</p> <p>Free radical formation by hepatic microsomal cytochrome P-450 reduction of <u>gen-tian violet</u>, <u>SO₂</u>, <u>CCl₄</u> and O₂ has also been investigated.</p>		

A. Radical Anion Metabolites

Nature of Problem: Before free radical metabolism can clearly be implicated in the origin of any toxic effect, three conditions must be met. First, the free radical metabolite must be demonstrated to exist. The best method of detecting free radicals is that of electron spin resonance spectroscopy, which permits the determination of both the free radical's structure and concentration. Second, because the toxicity of free radicals is presumably the result of purely chemical reactions, a knowledge of the radical's chemistry under physiological conditions is necessary. The third condition is that the characteristics of the toxicity must be consistent with the known enzymatic and nonenzymatic free radical reactions. In some cases the manipulation of an animal model provides evidence that a toxicity is indeed free radical-mediated. Carbon-tetrachloride, paraquat, and nitrofurantoin poisoning represent examples of free radical-mediated toxicity which have been well studied and where all three of these conditions have been fulfilled.

Objectives: The objective of this research is first, to search for free radical metabolites and then, on the basis of what is known about the free radical chemistry, to investigate the biochemical and toxicological implications of these free radical reactions.

Recent Accomplishments: Nifurtimox and nitrofurantoin were reduced by intact rat liver mitochondria to nitro anion radicals whose auto-oxidation generates superoxide anion as detected by direct electron spin resonance spectroscopy and by spin trapping experiments, respectively. Although nitro-reduction occurred in the presence of respiratory substrates, such as β -hydroxybutyrate, malate-glutamate, succinate, or endogenous substrates, nitro anion radical formation was much greater on addition of exogenous reduced pyridine nucleotides. NAD(P)H generated from endogenous NAD(P)⁺ by intramitochondrial reactions could not be used for the NAD(P)H nitroreductase reactions unless the mitochondria were solubilized by detergent. It is concluded that the nitro reductase activity of respiratory chain enzymes is far less important than that of enzyme(s) located in the outer membrane.

In the past, studies of the anion radical metabolites formed by hepatic microsomal one-electron reduction implicated one-electron donation from NADPH-cytochrome P-450 reductase. More recently, cytochrome P-450 has been found to transfer one electron to toxic chemicals such as gentian violet, sulfur dioxide, and molecular oxygen. When gentian violet is metabolized under a nitrogen atmosphere by rat hepatic microsomes supplemented with NADPH, a single-line ESR spectrum is obtained. Either CO or metyrapone inhibits radical formation by 50%, suggesting cytochrome P-450 involvement. Under an atmosphere of nitrogen, rat liver microsomal incubations containing bisulfite (aqueous sulfur dioxide) and NADPH form a free radical with a single-line ESR spectrum. These results imply that cytochrome P-450 reduces bisulfite to the sulfur dioxide anion radical. Oxygen completely inhibited the formation of this radical, which is

consistent with oxygen being a competitive inhibitor for the reduced heme of cytochrome P-450. We have used the spin trapping technique to demonstrate the microsomal reduction of oxygen to superoxide, which we also found to be inhibited by CO and metyrapone.

Experimental Approach: Free radicals may be detected, identified and quantitated by ESR spectroscopy. However, since most free radicals generated enzymatically are chemically reactive, they cannot always be observed by direct ESR. For such radicals the technique of spin trapping may be employed. This procedure uses diamagnetic organic molecules (spin traps) that react with free radicals to produce stable nitroxide radicals (spin adducts). The identity of the parent radical may often be determined from the ESR spectrum of the resultant spin adduct.

B. Plans for Subsequent Year

Previous studies have shown that subcellular systems, e.g., microsomal cytochrome P-450 and prostaglandin synthase, as well as purified enzymes, e.g., the peroxidases, form free radical metabolites from a wide variety of organic compounds. However, very few attempts have been made to detect free radicals in cellular systems.

We will use electron spin resonance techniques to search for free radical metabolites in hepatocytes. None of the many known radical anion metabolites has ever been studied in this system with electron spin resonance. Using other, less direct approaches, menadione is perhaps the best studied of these radical anion precursors. Electron spin resonance studies of this system should be the easiest place to start an investigation of anion radical metabolite formation by hepatocytes. We will also collaborate with Professor Thurman in spin trapping studies of CCl₄ metabolites in the perfused liver system.

C. Publications of past 18 months

Mason, R.P.: Assay of in situ radicals by electron spin resonance. In Packer, L. (Ed): Oxygen Radicals in Biological Systems, Methods in Enzymology, 105, New York Academic Press, 1984, pp. 416-422.

Kalyanaraman, B., Mottley, C. and Mason, R.P.: On the use of organic extraction in the spin-trapping technique as applied to biological systems. J. Biochem. Biophys. Meth. 9: 27-31, 1984.

Moreno, S.N.J., Mason, R.P., and Docampo, R.: Reduction of nifurtimox and nitrofurantoin to free radical metabolites by rat liver mitochondria. Evidence of an outer membrane-located nitroreductase. J. Biol. Chem. 259: 6298-6305, 1984.

Moreno, S.N.J., Mason, R.P., and Docampo, R.: Ca²⁺ and Mg²⁺ -enhanced reduction of arsenazo III to its anion free radical metabolite and generation of superoxide anion by an outer mitochondrial membrane azoreductase. J. Biol. Chem. 259: 14609-14616, 1984.

Polnaszek, C.F., Peterson, F.J., Holtzman, J.L., and Mason, R.P.: No detectable reaction of the anion radical metabolite of nitrofurans with reduced glutathione or macromolecules. Chem.-Biol. Inter. 51: 263-271, 1984.

Moreno, S.N.J., Mason, R.P., and Docampo, R.: Reduction of the metallochromic indicators arsenazo III and antipyrilazo III to their free radical metabolites by cytoplasmic enzymes. FEBS Letters 180: 229-233, 1985.

Mason, R.P. and Josephy, P.D.: Free radical mechanism of nitroreductase. In Rickert, D. (Ed): Toxicity of Nitroaromatic Compounds, New York Hemisphere, 1985, pp. 121-140.

Josephy, P.D. and Mason, R.P.: Nitroimidazoles; In Anders, M.W. (Ed): Bioactivation of Foreign Compounds, New York, Academic Press, 1985, pp. 451-483.

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Mason, R.P., and Josephy, P.D.: An electron spin resonance investigation of the iron-catalyzed reaction of metronidazole with cysteine. J. Inorg. Biochem. (in press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 50079-03 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Free Radical Metabolite Formation by Peroxidases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Ronald P. Mason	Research Chemist	LMB	NIEHS
OTHER:	Klaus Stolze	Visiting Fellow	LMB	NIEHS
	Carolyn Mottley	IPA	LMB	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Molecular Biophysics

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.0

PROFESSIONAL

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The objectives of this investigation are to detect, quantitate and identify free radical metabolites generated from endogenous and exogenous chemicals by peroxidase enzymes. Electron spin resonance (ESR) investigations of the prostaglandin hydroperoxidase and a model enzyme system, horseradish peroxidase, have demonstrated the enzymatic formation of free radical metabolites. The oxidation of benzidine was studied using horseradish peroxidase and prostaglandin synthase. Benzidine was metabolized to a radical cation and a charge-transfer complex composed of the benzidine and its two-electron (di-imine) oxidation product. The di-imine is a resonance structure of the nitrenium ion, the proposed ultimate carcinogenic metabolite of aromatic amines. ESR, on a millisecond time scale, has revealed the formation of a transient phenoxyl radical in the reaction of acetaminophen with horseradish peroxidase/H₂O₂ and bovine lactoperoxidase/H₂O₂. The short-lived radical is clearly distinguished from the persistent paramagnetic melanin polymers that are generated by prolonged incubation of acetaminophen in the presence of oxidizing enzymes. Ram seminal vesicles and acetaminophen under fast-flow conditions demonstrated the oxidation of acetaminophen to its phenoxyl free radical by the mammalian enzyme prostaglandin hydroperoxidase. Sulfur-centered free radicals have been detected when cysteine was incubated with horseradish peroxidase and H₂O₂. In the presence of either molecular oxygen or hydrogen peroxide, the thiyl radical is converted to the cysteine sulfonic and sulfinic acids. Reduced glutathione (GSH) was also oxidized to a sulfur-centered radical (GS·) by horseradish peroxidase and H₂O₂. Since cysteine and glutathione play an important role in the structure and function of sulfhydryl-containing proteins, these oxidation reactions may modulate the biological function of these compounds.

A. Free Radical Metabolite Formation by Peroxidases

Nature of Problem: Free-radical intermediates have been found in the metabolic pathways of a wide variety of organic compounds, and, on the whole, free radical metabolites seem to be implicated in the toxic effects of those xenobiotics which are metabolized to free radicals. In general, these toxic effects appear to be the result of the purely chemical reactions of the free radical; therefore, a knowledge of the chemistry of the free radical, as well as a demonstration of its existence in the metabolic pathway, is necessary before free-radical metabolism can be implicated in the origin of a toxic effect. In a few cases animal model studies have indicated that the toxicity of a xenobiotic is indeed free-radical mediated.

Objectives: The objectives of this project are: (a) to use electron spin resonance (ESR) and other techniques to detect, identify and quantitate free radical metabolites generated by the metabolism of endogenous and exogenous compounds; (b) to implicate free radical metabolites in the known toxic effects of the parent chemical; and (c) to determine the biological role played by free radicals derived from endogenous compounds.

Experimental Approach: Free radicals may be detected, identified and quantitated by ESR spectroscopy. However, since most free radicals generated enzymatically are chemically reactive, they cannot always be observed by direct ESR. For such radicals the technique of spin trapping may be employed. This procedure uses diamagnetic organic molecules (spin traps) that react with free radicals to produce stable nitroxide radicals (spin adducts). The identity of the parent radical may often be determined from the ESR spectrum of the resultant spin adduct.

Recent Accomplishments and Significance:

Acetaminophen: There is considerable evidence for the in vivo oxidation of acetaminophen to an arylating intermediate, N-acetyl-p-benzo-quinoneimine, which may bind to tissue macromolecules and cause hepatic necrosis. We have demonstrated by fast-flow electron spin resonance spectroscopy that the horseradish peroxidase/hydrogen peroxide system oxidized acetaminophen to a transient phenoxyl free radical, which is thought to be the intermediate in the oxidation of acetaminophen to N-acetyl-p-benzoquinoneimine. Both an overmodulated and high resolution spectrum have been obtained under fast-flow conditions. A more stable derivative of acetaminophen, with methyl groups placed at the ortho positions of the oxygen-centered free radical, has been prepared and used to study the dependence of free radical formation on enzyme and substrate concentration. The enzymatic oxidation of 3,5-dimethylacetaminophen with the horseradish peroxidase/hydrogen peroxide system forms a phenoxyl free radical metabolite. The structure of this free radical was established by a complete analysis of the ESR spectrum and confirmed by deuterium isotope substitution. Concomitant with phenoxyl radical formation, N-acetyl-3,5-dimethyl-p-benzoquinone imine was detected by optical spectroscopy. The phenoxy free radical was also formed by comproportionation in solutions of the quinone imine and added 3,5-dimethylacetaminophen. In contrast to acetaminophen, these imine and radical metabolites are stable and can be detected without resort to rapid-mixing techniques. The high reactivity of the free radical metabolite of acetaminophen may be related to the toxicity of this drug in vivo.

Cysteine: The sulfhydryl group of cysteine plays many important roles in both the structure and function of proteins, and the oxidation of L-cysteine often modulates these roles. The oxidation of L-cysteine is known to be responsible for the radioprotection of intracellular GSH, the bactericidal effect of cysteine, and the loss of ATP and GSH from isolated rat hepatocytes. Although the importance of free radicals in the radiolytic and metal ion oxidation of L-cysteine is clear, no direct evidence of a role for cysteine-derived free radicals in an enzymatic reaction has been reported. We have used the spin-trapping ESR technique to study free radical metabolites formed via the oxidation of cysteine by the peroxidase prototype, horseradish peroxidase. The oxidation of L-cysteine by horseradish peroxidase in the presence of oxygen forms a thiyl free radical, which reacts with spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) to form the DMPO-cysteine thiyl radical adduct. This radical adduct has a distinctive ESR spectrum ($a_N = 15.3$ and $a_H = 17.0$ G). Omission of hydrogen peroxide under anaerobic conditions results in a dramatic decrease in signal intensity, indicating that hydrogen peroxide is necessary for the adduct to form anaerobically. However, aerobic incubations without added hydrogen peroxide show signal intensities comparable to those obtained anaerobically with hydrogen peroxide. Thus, it appears that oxidation of L-cysteine by horseradish peroxidase to the corresponding thiyl free radical is dependent upon peroxide(s) that can be formed from atmospheric oxygen. With addition of catalase, 80% inhibition of adduct accumulation was obtained. Heat-denatured catalase had no effect. Studies with a Clark oxygen electrode show that oxygen is consumed in incubations of cysteine and horseradish peroxidase, presumably due to reactions of the thiyl free radical. The oxygen consumption is inhibited by DMPO as expected. Addition of molecular oxygen or hydrogen peroxide to the thiyl radical ultimately leads to cysteine sulfinic and cysteine sulfonic acids. The oxidation of cysteine sulfinic acid leads to the formation of both sulfur-centered and carbon-centered free radicals. GSH is also oxidized to GS[•] by horseradish peroxidase or prostaglandin hydroperoxidase and hydrogen peroxide, but not by glutathione peroxidase and hydrogen peroxide. Thiyl radical formation by horseradish peroxidase is inhibited by glutathione peroxidase after a rapid stimulation phase.

B. Plans for Subsequent Year

This work is being extended to sulfhydryl drugs used to treat hyperthyroidism. The mechanism of action of these drugs is thought to involve thyroid peroxidase. The oxidation of GSH by prostaglandin hydroperoxidase is being considered as the endogenous reducing cofactor responsible for the conversion of PGG₂ to PGH₂.

C. Publications of past 18 months

Harman, L.S., Mottley, C. and Mason, R.P.: Free radical metabolites of L-cysteine oxidation. J. Biol. Chem. 259: 5606-5611, 1984.

West, P.R., Harman, L.S., Josephy, P.D., and Mason, R.P.: Acetaminophen: enzymatic formation of a transient phenoxyl free radical. Biochem. Pharm. 33: 2933-2936, 1984.

Fischer, V., and Mason, R.P.: Stable free radicals and benzoquinone imine metabolites of an acetaminophen analogue. J. Biol. Chem. 259: 10284-10288, 1984.

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Eling, T.E., Reed, G.A., Krauss, R.S., Mason, R.P., and Boyd, J.A.: Metabolism of carcinogens by prostaglandin H synthase. In Thaler-Dao, et al. (eds): Icosanoid and Cancer, Raven Press, New York, pp. 63-70, 1984.

Mason, R.P., Chignell, C.F., Eling, T.E., and Mottley, C.: Free radical formation during the peroxidase-catalyzed oxidation of (bi)sulfite (hydrated sulfur dioxide). In Michelson, A.M. and Bannister, J.V. (eds): Life Chemistry Reports Supplement 2, Harwood Academic Publishers, Chur, pp. 55-63, 1984.

Mason, R.P., Harman, L.S., and Mottley, C.: Free radical metabolites of L-cysteine and glutathione oxidation. In Paton, W., Mitchell, J., and Turner P. (eds): IUPHAR 9th International Congress of Pharmacology, Vol. 2, MacMillan Press, London, pp. 233-241, 1984.

Eling, T., Mason, R. and Sivarajah, K.: The formation of aminopyrine cation radical by the peroxidase activity of prostaglandin H synthase and subsequent reactions of the radical. J. Biol. Chem. 260: 1601-1607, 1985.

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Fischer, V., West, P.R., Nelson, S.D., Harvison, P.J., and Mason, R.P.: Formation of 4-aminophenoxy free radical from the acetaminophen metabolite N-acetyl-p-benzoquinone imine. J. Biol. Chem. (in press).

Fischer, V., Harman, L.S., West, P.R., and Mason, R.P.: Free radical metabolites of acetaminophen and its methylated derivatives. Environ. Health Perspect., (in press).

Sealy, R.C., Harman, L.S., West, P.R., and Mason, R.P.: The electron spin resonance spectrum of the tyrosyl radical. J. Am. Chem. Soc. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 50080-03 LMB																												
PERIOD COVERED October 1, 1984 to September 30, 1985																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Environmental Health Applications of Mass Spectrometry																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">Simon J. Gaskell</td> <td style="width: 33%;">Expert</td> <td style="width: 33%;">LMB</td> </tr> <tr> <td colspan="4" style="padding-top: 10px;">OTHER:</td> </tr> <tr> <td>Donald Harvan</td> <td>Chemist</td> <td></td> <td>LMB</td> </tr> <tr> <td>Gordon Dean Marbury</td> <td>Chemist</td> <td></td> <td>LMB</td> </tr> <tr> <td>Carol E. Parker</td> <td>Chemist</td> <td></td> <td>LMB</td> </tr> <tr> <td>Donald J. Harvan</td> <td>Chemist</td> <td></td> <td>LMB</td> </tr> <tr> <td>Richard Smith</td> <td>Chemist</td> <td></td> <td>LMB</td> </tr> </table>			PI:	Simon J. Gaskell	Expert	LMB	OTHER:				Donald Harvan	Chemist		LMB	Gordon Dean Marbury	Chemist		LMB	Carol E. Parker	Chemist		LMB	Donald J. Harvan	Chemist		LMB	Richard Smith	Chemist		LMB
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COOPERATING UNITS (if any)																														
LAB/BRANCH Laboratory of Molecular Biophysics																														
SECTION Mass Spectrometry																														
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																														
TOTAL MAN-YEARS 1.15	PROFESSIONAL 0.9	OTHER 0.25																												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews																					
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<input type="checkbox"/> (a2) Interviews																														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Approximately 700 samples have been analyzed as part of the service and collaborative support program. The techniques employed spanned the full range of mass spectrometric methods and instrumentation available to the mass spectrometry group. Collaborative projects included applications of combined liquid chromatography-mass spectrometry, contributions to drug metabolism studies and the use of tandem mass spectrometry in structure elucidation.																														

A. Environmental Health Applications of Mass Spectrometry

Nature of Problem: Substantial contributions to the structure elucidation of complex biomolecules and to the determination of components of complex mixtures of biological origin may be made by application of sophisticated mass spectrometric techniques.

Objectives: To apply state-of-the-art mass spectrometry to analytical problems presented by coworkers at NIEHS and collaborators from outside institutions.

Experimental Approach: Various forms of mass spectrometry are used, including a variety of ion sources - electron impact, chemical ionization, fast atom bombardment, thermospray. Reagent gases used for chemical ionization include methane, isobutane and ammonia. Positive and negative ion detection are available. Inlet systems include direct insertion probe, gas chromatograph and liquid chromatograph. Most of the service support is provided using a magnetic sector, double-focussing mass spectrometer but liquid chromatography-mass spectrometry is performed on quadrupole instruments. A 'bench-top' instrument, the Finnigan Ion Trap Detector (of quadrupole design), has been recently installed and is currently being evaluated for routine quantitative analyses.

Recent Accomplishments:

Service support: Approximately 700 samples have been analyzed; of these, about 52% were direct probe analyses, 24% involved gas chromatography-mass spectrometry, 8% employed fast atom bombardment and 16% were quantitative analyses by selected ion monitoring. Over half of the direct probe and fast atom bombardment analyses, and all of the quantitative analyses, were related to the research of NIEHS scientists from outside the Laboratory of Molecular Biophysics.

Liquid chromatography-mass spectrometry (LC-MS) of analogues of diethylstilbestrol (with L. Levy, LMB, and K. Korach, LRDT): Thermospray LC-MS has been used to separate and identify analogues of stilbestrol, including (through the use of a chiral stationary phase) enantiomers.

Identification of the monoglucuronide of Santonox in male rats (with H. Matthews, LRDT): This was achieved by a combination of LC-MS and fast atom bombardment techniques.

Characterization of the major rat urinary metabolite of 2,4-dinitroaniline (with H. Matthews, LRDT): Analyses by LC-MS indicated the structure to be 2,4-dinitrophenylhydroxylamine, excreted as the sulfate and glucuronide conjugates.

Characterization of a peptide (PLIM) using tandem high resolution mass spectrometry with collisional-activation techniques (with W. Wilson, LBNT).

Analyses of steroid glucuronides (with CHL Shackleton, Oakland Children's Hospital, Oakland, CA): Tandem mass spectrometry has been used to overcome the limitations of conventional techniques in the differentiation of isomeric

(including stereoisomeric) steroid glucuronides. Such distinctions are important in the characterization of certain metabolic disorders.

B. Plans for subsequent year:

The research support program will continue. In particular, more emphasis will be placed on the availability to colleagues and collaborators of new analytical techniques developed during the course of other research projects within the Mass Spectrometry Group.

C. Publications of last 18 months:

Parker, C.E., Levy, L.A., Smith, R.W., Yamaguchi, K., Gaskell, S.J., and Korach, K.S.: Separation and detection of enantiomers of stilbestrol analogues by combined liquid chromatography/thermospray mass spectrometry. J. Chromatog. (In press).

Smith, R.W., Parker, C.E., Matthews, H.B., and Hass, J.R.: Identification of the major metabolite of 4,4'-thio-bis-(6-t-butyl-m-cresol). Biomed. Mass Spectrom. 12: 208-214, 1985.

Ayanoglu, E., Wegmann, A., Pile, O., Marbury, G.D., Hass, J.R., and Djerassi, C.: Mass spectrometry of phospholipids. Some applications of desorption chemical ionization and fast atom bombardment. J. Am. Chem. Soc. 106: 5246-5251, 1984.

Albro, P.W., Corbett, J.T., Marbury, G.D., and Parker, C.E.: Urinary metabolites of orally administered di-(5-hexenyl) phthalate and di-(9-decenyl) phthalate in the rat. Xenobiotica 14: 389-398, 1984.

Tondeur, Y., Hass, J.R., Harvan, D.J., McKinney, J.D., and Albro, P.W.: Determination of suspected toxic impurities in firemaster FF-1 or BP-6 by capillary column gas chromatography/high resolution mass spectrometry. J. Ag. Food Chem. 32: 406-410, 1984.

Parker, C.E., Albro, P.W., Abusteit, E.O., Mester, T.C., Hass, J.R., Sheldon, Y.S., and Corbin, F.T.: Determination of the pKa values of metribuzin and its metabolites: A comparison of spectrophotometric and titrimetric methods. J. Ag. Food Chem. 32: 212-217, 1984.

Tondeur, Y., Albro, P.W., Hass, J.R., Harvan, D.J., and Schroeder, J.L.: Matrix effects and sub-part-per-billion quantitative analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin by mass spectrometry. Anal. Chem. 56: 1344-1347, 1984.

Albro, P.W., Parker, C.E., Marbury, G.D., Hernandez, O., and Corbin, F.T.: Spectrometric investigations of metribuzin and its metabolites. Appl. Spectrosc. 38: 556-562, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 50082-02 LMB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies on Tumor Promoters and Antipromoters		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Phillip W. Albro Research Chemist LMB NIEHS Other: Ram Rudra Shukla Visiting Fellow LMB NIEHS		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Molecular Biophysics		
SECTION Bio-organic Chemistry		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: <div style="text-align: center;">1.8</div>	PROFESSIONAL: <div style="text-align: center;">0.8</div>	OTHER: <div style="text-align: center;">1.0</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> <u>Tumor promoters</u> can often be differentiated by their effects in two model systems: <u>polynuclear aromatic hydrocarbon (PAH)-initiated carcinogenesis</u> in mouse skin and <u>nitrosamine-initiated carcinogenesis</u> in regenerating rat or mouse liver. <u>Phorbol esters</u> promote in both systems, some <u>phthalate esters</u> promote the latter and fail to affect the former, <u>2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)</u> promotes the latter and is an antipromoter for the former, while <u>polychlorinated biphenyls (PCBs)</u> may promote or act as an antipromoter for both depending on the dosages tested. A comparison of these classes of environmental pollutants may provide mechanistic insights absent when any one is studied by itself. Initial studies indicate that one must expect the effects of these compounds on given model systems (such as <u>lipid peroxidation</u>) to be significantly different <u>in vitro</u> from <u>in vivo</u>. </p>		

A. Studies on Tumor Promoters and Antipromoters

Nature of Problem: Several classes of common environmental pollutants are positive in model system tests for tumor promotion but negative in tests for initiation, genotoxicity, and mutagenesis. Some of these classes of compounds, for example chlorinated dibenzo-p-dioxin, polychlorinated biphenyls, and phthalate diesters, also act as antipromoters in other protocols, antagonizing the promotion by classical promoters such as the phorbol esters. This dichotomy makes it conceptually difficult to visualize the mechanism(s) of action of a member of these classes.

Objectives: (1) Considering the chemical classes polychlorinated dibenzo-p-dioxins (esp. 2,3,7,8-TCDD), polychlorinated biphenyls, phorbol esters and phthalate diesters (esp. DEHP), to identify biochemical systems with which more than one class interacts and which may have some relationship to promotion or antipromotion; (2) to form and test hypotheses concerning the biochemical or molecular level mechanisms by which these chemicals exert their promoter or antipromoter activity. More specific objectives will be defined as the project develops.

Experimental Approach: Since there are already a number of systems with which phorbol ester promoters are known to interact, including specific receptors, lipid peroxidation in target tissues, specific hydrolase enzymes related to species differences in susceptibility, effects on T-killer cell production (immune surveillance), and several others, the other classes of test compounds will be tested for ability to interact with these model systems. In most cases radioisotopically labeled forms of the test compounds are used to measure binding, metabolic alteration, etc. Possible effects of the test compounds on metabolic activation of initiator compounds are investigated using radiolabeled initiators in tests of covalent binding to macromolecules. Lipid peroxidation is studied by ESR spectrometry, measurement of aldehyde production, conjugation of double bonds, disappearance of polyunsaturated fatty acids, and production of fluorescent pigments.

Recent Accomplishments and Significance: One phenomenon common to all the promoters under study is the in vivo stimulation of lipid peroxidation. This is not presently thought to be directly involved in the process of tumorigenesis, but rather to be an indicator of the increased production of activated (radical?) species, possibly including some that do play a role in the promotion process. A hypothesis has been presented in the literature suggesting that the peroxidation associated with exposure to TCDD in some way results from, and requires, the induction of microsomal cytochrome P-448-dependent mixed function oxidase activity by TCDD. However, we have been able to demonstrate a stimulating effect of TCDD at the low nanogram per ml level and below on microsomal lipid peroxidation in vitro, under conditions where enzyme induction is impossible. This effect shows an absolute dependence on chelated iron, and strong species specificity, and implies that induction of oxidase enzymes may not be necessary for TCDD to exert this type of biological effect.

B. Plans for Subsequent Year

1. Presently available techniques for the study of peroxidative events, especially lipid peroxidation, are very effective for in vitro studies but insensitive and generally uninformative relative to peroxidative events that occurred in vivo. We also plan to investigate the potential for, and mechanisms of, repair of peroxidative damage in tissues. Such mechanisms clearly do exist, but have been little studied.

2. Most of the tumor promoters of the types being studied are known to be capable of inducing the synthesis of (usually microsomal) enzymes in rodent livers. Two classes of enzymes, those involved in methylation of DNA during cell differentiation and those involved in phosphorylation of proteins, may arguably play a role in carcinogenesis. The ability of the tumor promoters to induce these types of enzymes will be investigated. Should the results be positive, we will consider developing immunoassays for the specific enzymes of interest.

Publications

Chae, K., Albro, P.W., Luster, M.I. and McKinney, J.D.: Screening assay for the tetrachlorodibenzo-p-dioxin receptor using the 125-I-Iodovaleramide derivative of trichlorodibenzo-p-dioxin as the binding ligand.

Int. J. Environ. Anal. Chem. 17: 267-274, 1984.

Rumbaugh, R.C., McCoy, Z., Hass, R., Harvan, D., Albro, P.W., and Lucier, G.W.: Induction of hepatic microsomal aryl hydrocarbon hydroxylase in Sprague-Dawley rats by administration of soil contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxide. Toxicol. Appl. Pharmacol. (in press)

Albro, P.W., Schroeder, J.S., Harvan, D.J., and Corbett, J.T.: Characteristics of an extraction and purification procedure for chlorinated dibenzo-p-dioxins and dibenzofurans in soil and liver. J. Chromatogr. 312: 165-182, 1984.

Bend, J.R., Foureman, G.L., Ben-Zvi, Z., and Albro, P.W.: Heterogeneity of hepatic aryl hydrocarbon hydroxylase activity in feral winter flounder: Relevance to carcinogenesis. Natl. Cancer Inst. Monographs 65: 359-370, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 50083-01 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Specific Binding of Halogenated Aromatic Hydrocarbons to Thyroxine Binding Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: James D. McKinney Supervisory Research Chemist LMB NIEHS

OTHER: Kun Chae	Chemist	LMB	NIEHS
Urs Rickenbacher	Visiting Fellow	LMB	NIEHS
Ricky Fannin	Chemist	LMB	NIEHS
Sandy Jordan	Biologist	LMB	NIEHS

COOPERATING UNITS (if any)

Biochemical Toxicology Group, BRAP, NIEHS
Toxicology Research and Testing Program, NTP, NIEHS

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Biochemical/Toxicology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.5

PROFESSIONAL

1.5

OTHER

1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this work is to study the role of thyroid hormone binding proteins in mediating the toxic effects of certain halogenated aromatic hydrocarbons of environmental importance. Current interest is in the binding proteins specific for thyroxine (T₄). Prealbumin (TBPA) is a major thyroxine binding protein in blood which has been proposed as a model for the thyroxine nuclear receptor in tissue. Molecular interactions of TBPA with the dioxins, furans and polychlorinated biphenyls (PCBs) have been studied with use of computer graphics and predictions made regarding relative binding affinities for such structures. These modeling predictions were tested by experimentally measuring the binding affinities of soluble derivatives of those structures, and the results are in good agreement with prediction. The binding model can account for the requirement for lateral halogens and an approximately rectangular molecular shape in toxicity. Similar studies with the thyroxine nuclear protein solubilized from rat liver tissue also show competitive binding interactions and with similar binding specificities. A soluble dioxin approximate isostere shows a remarkably high affinity (half-maximal of 15 pM) for the nuclear receptor (compared to a half maximal of 15 nM for T₄). Thus the nuclear receptor affinity has the expected sensitivity for possible involvement in toxicity. Dose-dependent regulation (increase) of the T₄ nuclear receptor number by dioxin was demonstrated and suggest a possible mechanism for potent and persistent expression of thyroid hormone activity which could result in toxicity.

PROJECT DESCRIPTION

A. Specific Binding of Halogenated Aromatic Hydrocarbons to Thyroxine Binding Proteins The halogenated aromatic hydrocarbons are a class of chemicals to which essentially everyone is chronically exposed at low levels and to which some people are occupationally exposed at relatively high levels. Compounds studied include members of the biphenyl (PCBs, PBBs) naphthalene (PCNs, PBNs), dibenzo-p-dioxin (CDDs) and dibenzofuran (CDFs) chemical classes. Among these classes are some of the most toxic small molecules known. Dermatological effects, including chloracne, and an increase in liver enzyme activity have been clearly demonstrated in human populations. However, health effects in the areas of hepatotoxicity, mutagenesis, and reproductive effects are still the object of a continuing controversy. Since our laboratory has been interested in the chemistry and biochemical toxicology of these compounds for many years and has developed techniques used in their study, we continue to focus our attention on the halogenated aromatic hydrocarbons as model compounds for accomplishing research to meet our overall program objectives. Specifically we hope to determine the structural basis of dioxin and related compound toxicity and compare it with the same observed for other kinds of biological activity such as mixed function oxidase induction. This information in turn should be useful in assessing toxicologically relevant protein receptors in biological systems which could be further studied to delineate their role in the mechanisms of toxicity. In the course of this work, we will develop our research hypotheses and conclusions in terms of molecular structure and theoretical chemistry which should provide a basis for making predictions concerning the toxic effects of untested compounds.

Objectives: The objective of this work is to study the role of thyroid hormone binding proteins in mediating the toxic effects of certain halogenated aromatic hydrocarbons of environmental importance. Current interest is in the binding proteins specific for thyroxine (T₄).

Experimental Approach and Scientific Justification: Competition binding assay methods involving purified thyroxine binding proteins [thyroxine binding prealbumin (TBPA) or solubilized nuclear protein preparations from rat liver] and ¹²⁵I-thyroxine are being used to study the structurally specific binding of selected halogenated aromatic hydrocarbons of environmental importance. This is being done (where possible) in association with molecular modeling work involving X-ray and minimum energy structures to enable predictions to be made with regard to untested compounds.

Recent Accomplishments and Significance to Biomedical Research: A number of compounds contained in the broad class of halogenated aromatic hydrocarbons including the PCBs, dioxins and furans produce a characteristic toxic syndrome. A unifying hypothesis for the molecular mechanism of action of these compounds has been proposed based on their potential to function as potent and resistant thyroxine agonists (or antagonists). Studies on the molecular interactions of these toxic compounds with thyroxine specific binding proteins should yield

information which can be used to test this hypothesis and elucidate the mechanism at the biochemical level. The interactions of 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds with prealbumin, a model for the nuclear thyroid hormone receptor, have been studied with use of computer graphics and predictions made regarding the binding affinities of dioxin and furan analogues. The results were in general agreement with the modeling predictions and demonstrated that such compounds could be effective competitive binding ligands for thyroxine-specific binding sites in prealbumin. The computer modeling work also demonstrates the importance of lateral chlorine substitution in the binding of these toxic compounds.

Study of the structurally specific binding of polychlorinated biphenyls (PCBs) to serum binding proteins specific for T_4 was of interest since these proteins may be involved directly (or indirectly) in mediating the goitrogenic and possibly other toxic effects of these environmentally important compounds. Prealbumin is a major thyroxine binding protein in blood that has been well studied crystallographically. The interactions of polychlorinated biphenyls (PCBs) and related compounds with prealbumin have been studied with use of computer graphics and predictions made regarding relative binding affinities for such structures. These modeling predictions were tested by experimentally measuring the binding affinities of selected PCBs and hydroxylated derivatives. The results are in excellent agreement with the modeling predictions and demonstrated that these compounds can be highly effective (three to eight times better than thyroxine itself) competitive binding ligands for thyroxine specific binding sites in prealbumin. Our finding that laterally (3,3',5,5'-) substituted PCBs show the highest binding activity and that further substitution on nonlateral (2,2',6,6'-) positions may lower binding activity is substantially consistent with the structure-toxicity relationship for these compounds. Thus, the binding model can account for the requirement for lateral halogens in toxicity and may also suggest a need for an approximately rectangular molecular shape. This work extends the use of the prealbumin interaction model for studying the structure-toxicity relationship of halogenated aromatic hydrocarbons compounds. In addition, the presence of certain PCBs and related compounds in blood is likely to significantly alter hormone-protein interactions which are responsible for the maintenance of normal thyroid status and may also modulate the distribution of certain PCBs in the body. Since prealbumin is also a model for the putative thyroxine nuclear receptor in tissue, our hypothesis that high toxicity of certain halogenated aromatic hydrocarbons is the expression of potent and persistent thyroid hormone activity (responses induced by certain halogenated aromatic hydrocarbons which qualitatively correspond to those mediated by thyroid hormones) is further supported.

A reproducible binding assay for the solubilized thyroxine specific binding protein in rat liver tissue has been developed. The thyroxine nuclear receptor in rat liver tissue shows a binding specificity for chlorinated aromatic hydrocarbons that depends on lateral halogenation. Binding affinity is significantly greater for structures that also have a linear shape. Lateral halogenation and a linear shape are also characteristic of highly toxic molecules of this type. A soluble dioxin approximate isostere shows a remarkably high affinity

(half-maximal of 15 pM) for the nuclear receptor (compared to a half-maximal of 15 nM for T₄). Thus, the nuclear receptor affinity has the expected sensitivity for possible involvement in toxicity. Dose-dependent regulation (increase) of the T₄ nuclear receptor number by dioxin has been demonstrated (Scatchard analysis) and suggest a possible mechanism for potent and persistent expression of thyroid hormone activity which could result in toxicity.

Plans for Subsequent Year: In order to establish our PCB-TBPA interaction model for further investigations of this type, we are attempting to cocrystallize 3,3',5,5'-tetrachloro-4,4'-dihydroxybiphenyl and TBPA for X-ray study. This compound is very nearly isosteric and isoelectronic with the toxic 3,3',4,4',5,5'-hexachlorobiphenyl. We also hope to be able to study the PCB-TBPA system directly in solution using nuclear magnetic resonance (NMR) measurements. Other work will attempt to estimate binding enthalpies using molecular mechanics energy minimization procedures.

We also plan to develop an affinity column for the nuclear T₄ receptor which will enable its isolation and purification. Purified receptor protein will facilitate further studies which will attempt to relate ligand concentrations to both primary and ultimate (toxic) biochemical responses. Future in vivo studies will investigate the potential of dioxin and related toxic compounds to displace ¹²⁵I-T₄ from nuclear binding sites in a dose-dependent way. Related studies will examine the time course for dioxin induced T₄-nuclear protein receptor increase as a function of dose.

C. Publications of past 18 months

McKinney, J.D., Chae, K., Oatley, S.J., and Blake, C.C.F.: Molecular interactions of toxic chlorinated dibenzo-p-dioxins and dibenzofurans with thyroxine binding prealbumin. J. Med. Chem. 28: 375-381, 1985.

McKinney, J.D., Chae, K., Jordan, S., Luster, M., Tucker, A., and Oatley, S.: TCDD binds the nuclear receptor for thyroxine. Society of Toxicology Meeting, San Diego, CA, The Toxicologist 5: 803, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 50084-01 LMB																									
PERIOD COVERED October 1, 1984 to September 30, 1985																											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Modeling Approaches to the Study of Molecular Mechanisms of Toxic Action																											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">James D. McKinney</td> <td style="width: 30%;">Supervisory Research Chemist</td> <td style="width: 10%;">LMB</td> <td style="width: 10%;">NIEHS</td> </tr> <tr> <td>OTHER:</td> <td>Thomas Darden</td> <td>Computer Scientist</td> <td>BRAP</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>Andy Maynard</td> <td>Computational Chemist</td> <td>LMB</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>Lee Pederson</td> <td>Theoretical Chemist</td> <td>LMB</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>Herbert Posner</td> <td>Biochemist</td> <td>LMB</td> <td>NIEHS</td> </tr> </table>			PI:	James D. McKinney	Supervisory Research Chemist	LMB	NIEHS	OTHER:	Thomas Darden	Computer Scientist	BRAP	NIEHS		Andy Maynard	Computational Chemist	LMB	NIEHS		Lee Pederson	Theoretical Chemist	LMB	NIEHS		Herbert Posner	Biochemist	LMB	NIEHS
PI:	James D. McKinney	Supervisory Research Chemist	LMB	NIEHS																							
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	Lee Pederson	Theoretical Chemist	LMB	NIEHS																							
	Herbert Posner	Biochemist	LMB	NIEHS																							
COOPERATING UNITS (if any) Department of Chemistry, University of California, San Diego, CA Laboratory Molecular Biophysics, University of Oxford, England Department of Chemistry, University of North Carolina, Chapel Hill, NC																											
LAB/BRANCH Laboratory of Molecular Biophysics																											
SECTION Molecular/Theoretical Modeling																											
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																											
TOTAL MAN-YEARS: <div style="text-align: center;">3.5</div>	PROFESSIONAL: <div style="text-align: center;">2.5</div>	OTHER: <div style="text-align: center;">1</div>																									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																											
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> This program develops and maintains an <u>interconnected system of computer programs</u> which would allow a chemist or biologist to conveniently ask <u>theoretical questions about molecules of interest</u>. Using <u>structure-activity, molecular and theoretical modeling approaches</u>, we attempt to explain how structure determines toxicity and propose testable <u>mechanistic hypothesis for toxic action</u>. Our experience using the limited form in which our system now exists has amply proven the value of a unified approach, but the fast pace of hardware and software developments in this field, together with the complexity of the individual components has so far precluded the existence of a "ready made" solution to our needs. In association with an experimental group the program continues to investigate the molecular interaction of the <u>halogenated aromatic hydrocarbons</u>, an important class of toxic environmental agents. Early studies focusing on <u>polychlorinated biphenyl (PCBs)</u> and related compounds led to the development of a theoretical model for the <u>dioxin or Ah receptor</u> based on molecular parameters and <u>molecular mechanics</u>. Related studies with the chlorinated dioxins identified <u>common molecular properties with the thyroid hormones</u> which enable them to present a planar face and lateral halogens in interactions with proteins. Experimental studies are investigating the <u>binding behavior of PCBs with specific thyroxine binding proteins</u> along with <u>molecular dynamic simulation of these binding events</u>. In other work we are examining the use of <u>energy minimization programs</u> to accurately predict the energies and shapes of <u>metastable conformers of drugs and toxic chemicals</u>. A theoretical study of the minimum energy structures of <u>diethylstilbestrol and its analogs</u> by molecular mechanics (MM2P), MNDO and <u>ab initio calculations</u> found that MM2P agrees most closely with <u>X-ray</u> for all cases studied. </p>																											

PROJECT DESCRIPTION

A. Modeling Approaches to the Study of Molecular Mechanisms of Toxic Action

Nature of Problem: This program is using structure-activity, molecular and theoretical modeling approaches to elucidating mechanisms of biological action (with emphasis on toxicity). We attempt to explain how structure determines toxicity and propose testable mechanistic hypothesis for toxic action. Further, we hope to use structural and molecular/theoretical modeling insights to guide further biological experimentation aimed at understanding mechanisms of action at the molecular level. It is assumed that toxic endpoints are in some way associated with specific molecular interactions, probably involving specific receptor sites and that these interactions are the result of molecular reactivity properties encoded in the structure.

Objectives: Develop and evaluate the use of structure activity relationships in understanding molecular mechanisms of biological action for predicting the toxicological outcome. Develop systematically physical-organic measurements and calculations that characterize the structural properties important in these actions and explain how chemical structures determine the toxicological outcome. Use halogenated aromatic hydrocarbons as prototype compounds for these purposes.

Experimental Approach and Scientific Justification: An interconnected system of computational chemistry programs are developed and maintained to allow a chemist or biologist to conveniently ask theoretical questions about molecules of interest.

Recent accomplishments and Significance to Biomedical Research: The action of foreign compounds in biological systems can be produced by a variety of specific molecular interactions, and combinations of nonspecific processes. All of these actions reflect a close relationship between physicochemical properties encoded in the molecular structure of the compounds and the responses they evoke in biological systems. It is thus possible to seek an understanding of the structure-activity relationship for the toxic actions of foreign chemicals by trying to elucidate the relationship between molecular structure and effects on biological systems based on the properties encoded in the structures. Thus the molecular reactivity of these compounds is responsible for their recognition at biological acceptors and associated triggering of molecular mechanisms that lead to tissue response. It is assumed that the interaction between foreign compounds and biological targets is dependent on the same molecular properties that determine chemical interactions and reactions. Then, a description of these interactions and mechanisms should follow the formal patterns of chemistry modulated by the size and complexity of the biological environment.

Building a computer based system for modeling purposes involves procuring the appropriate pieces of hardware and software whenever possible, developing new software when this is necessary and feasible, learning enough about the separate pieces to facilitate communication of the pieces with the rest of the system and troubleshooting when problems manifest themselves.

The current hardware components of our system include the two NIEHS VAX 11/780's, and IRIS graphics workstation, a Rainbow 100 (on loan) for file transfers, a Tektronix 4107 graphics terminal with data tablet and ink-jet plotter, a Tektronix 4014 terminal with data tablet, screen dump and pen plotter and two VT240 terminals.

The separate programs we are using on the NIEHS Vax's include the ab initio quantum chemistry programs Gaussian 82 and UCSF Gaussian 80, the semi-empirical programs MOPAC, Forticon (for extended Huckel calculations) and the empirical force field program MM2, MM2p and QCFFPI (for small molecules) and RGDMIN, AMBER, and CHARMM (for macromolecules). The graphical molecule entry and energy minimization program MODEL has been extensively rewritten for the Tektronix 4107 to add the display programs PLUTO and MS, to allow for multimolecule docking interactions, and to serve as a front end driver for MM2p and AMBER. Eventually we plan to utilize MODEL as a front end for the other programs on the VAX as well as to coordinate communication with the IRIS. The separate programs we are using on the IRIS are MMS, FRODO and MIDAS. These programs provide graphical display and real time modeling interaction of macromolecules. We plan to interface these more tightly to the energy minimization programs we have available on the VAX and to adapt some of them to run on the IRIS. A version of MODEL for small molecules is also planned for the IRIS.

A study of the conformations of DES, and two pseudo-DES analogues EPD and ZPD was performed using Gaussian 82, MNDO and MM2p. These molecules are large compared to the typical molecule studied quantum mechanically and this study was an important benchmark for the strengths and limitations of the three separate approaches represented by these programs. In this study the theoretical energy minima were compared with each other and with the X-ray geometry. In order to discuss questions not easily accessible experimentally, such as the existence of metastable conformations, it is important to be able to easily ask these questions using a variety of theoretical approaches, and to understand the reasons for differences in the answers they give you. The value of a graphical display interface with regard to this last point has already been amply demonstrated. Further work on this study is currently in progress. Similar studies have compared the X-ray structure of selected PCBs with the energy minimized structures from both molecular mechanics and ab initio calculations.

Using the small molecule docking features of the modified MODEL program, a study of PCB interactions with porphine (as a model of the cytosol receptor) was carried out. Use of the solvent accessible surface and least-squares comparison features of the program allowed for further visual criteria of fit to be applied.

Present work centers on trying to understand the binding of hydroxylated PCB's to prealbumin. Binding data is available from our laboratory for a variety of these compounds. The X-ray structure of thyroxine bound to prealbumin is available courtesy of Dr. Stuart Oatley, (UCSD) who has also provided us with preliminary starting conformations for some of the PCB analogs using a graphical docking technique with FRODO. Another collaborator, Dr. Colin Blake, is

attempting to solve the X-ray structures for some of these compounds bound to prealbumin. We are currently trying to correlate computed energies and conformations of binding (using AMBER) with the experimental quantities mentioned above. The graphical display capabilities of the IRIS are crucial for the task because of the local minimum versus global minimum problems for a system such as this involving the order of a thousand degrees of freedom. We feel that this biological system provides us with a unique opportunity to understand receptor interactions in a case where the three dimensional structure of the receptor is available, binding experiments can be performed on promising analogs and the necessary software for modeling purposes is available.

Plans for Future: Further work will continue to stabilize and extend the interconnected system of computer programs available for molecular and theoretical modeling studies. We are also in the process of trying to gain access to the PROPHET system at NIH to further facilitate the molecular and theoretical modeling work.

We are also exploring the use of computer assisted pattern recognition methods in analyzing multivariate biological data bases to assist in identifying the different mechanisms of action that may be operating. In turn we are planning to develop theoretical models for each mechanism identified to assist in predicting the outcome of untested compounds. In preliminary studies, a homogenous set of mutagenic nitro aromatics have been identified for study. We hope to correlate mutagenic activity with their reduction potentials as estimated by their LUMO energies calculated by ab initio methods.

Other work will reexamine structural basis of polynuclear aromatic hydrocarbon carcinogenesis using molecular and theoretical modeling approaches.

Publications of past 18 months:

McKinney, J.D., Long, G.A., and Pederson, L.: PCB and dioxin binding to cytosol receptors: A theoretical model based on molecular parameters. Quant. Struct. Act. Relationships in Pharmacol. Chem. and Biol. 3: 99-105, 1984.

McKinney, J.D., Darden, T., Lyster, M.A., and Pederson, L.G.: PCB and related compound binding to the Ah receptor(s). Theoretical model based on molecular parameters and molecular mechanics. Quant. Struct. Act. Rel. in Pharmacol. Chem. and Biology accepted.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 50085-01 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Halogenated Aromatic Hydrocarbons as Thyroxine Agonists (or Antagonists)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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	Ricky Fannin	Chemist	LMB	NIEHS
	Sandy Jordan	Biologist	LMB	NIEHS

COOPERATING UNITS (if any)

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Duke Medical Center, Department of Radiology
Medical College of Wisconsin, Milwaukee

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Biochemical/Toxicology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2

PROFESSIONAL

1

OTHER

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

TCDD and thyroxine have common molecular reactivity properties which enable them to present a planar face and lateral halogens in interactions with proteins. These molecular properties are consistent with the structure-toxicity relationship of TCDD and related compounds. Biological evidence is discussed including preliminary studies on the effects of TCDD exposure on tadpole growth and development and on murine stem cell proliferation and differentiation which is consistent with the possible thyroxine-like activity of TCDD. The work suggests the possibility that toxicity is at least in part the expression of potent and persistent thyroid hormone activity (responses induced by TCDD which qualitatively correspond to those mediated by thyroid hormones). A mechanism for toxicity is proposed which involves receptor proteins; the planar aromatic system controls binding to cytosolic proteins and halogen substituents regulate binding to nuclear proteins. This simple model based on molecular reactivity sheds light on the diversified effects of TCDD and related compound toxicity and on certain thyroid hormone action. The model also permits predictions to be made with regard to the toxicity and thyroid hormone activity of untested compounds.

(Project No. Z01 ES 30066-09 LMB, "Structural Theoretical Basis and Molecular Mechanisms of Biological Action" is being continued under this Project No.)

PROJECT DESCRIPTION

A. Halogenated Aromatic Hydrocarbons as Thyroxine Agonists (or Antagonists)

Nature of Problem: The halogenated aromatic hydrocarbons are a class of chemicals to which essentially everyone is chronically exposed at low levels and to which some people are occupationally exposed at relatively high levels.

Compounds studied include members of the biphenyl (PCBs, PBBs) naphthalene (PCNs, PBNs), dibenzo-p-dioxin (CDDs) and dibenzofuran (CDFs) chemical classes.

Among these classes are some of the most toxic small molecules known.

Dermatological effects, including chloracne, and an increase in liver enzyme activity have been clearly demonstrated in human populations. However, health effects in the areas of hepatotoxicity, mutagenesis, and reproductive effects are still the object of a continuing controversy. Since our laboratory has been interested in the chemistry and biochemical toxicology of these compounds for many years and has developed techniques used in their study, we continue to focus our attention on the halogenated aromatic hydrocarbons as model compounds for accomplishing research to meet our overall program objectives. Specifically we hope to determine the structural basis of dioxin and related compound toxicity and compare it with the same observed for other kinds of biological activity such as mixed function oxidase induction. This information in turn should be useful in assessing toxicologically relevant protein receptors in biological systems which could be further studied to delineate their role in the mechanisms of toxicity. In the course of this work, we will develop our research hypotheses and conclusions in terms of molecular structure and theoretical chemistry which should provide a basis for making predictions concerning the toxic effects of untested compounds.

Objectives: In collaboration with toxicological scientists, we are testing the possibility that TCDD can function as a thyroxine agonist and interact directly with relevant tissue receptors where thyroid hormone activity may regulate gene expression.

Experimental Approach and Scientific Justification: A variety of methods exists for measuring thyroid hormone activity for thyroid hormone analogs and toxicity for halogenated aromatic hydrocarbons. We have to identify those methods which are best suited to show that thyroxine and dioxin can produce the same or similar effects in biological systems. Both qualitative and quantitative effects are of interest.

Recent Accomplishments and Significance to Biomedical Research: The halogenated aromatic hydrocarbons constitute a broad class of environmentally important compounds with varying structure and toxicity. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (dioxin or TCDD) the prototypical structure is the most toxic compound of this type and is also a potent inducer of cytochrome P-448 mediated mixed function oxidase enzyme systems. The mechanism of toxicity of these compounds has been under intense study in a number of laboratories throughout the world. In this work, we offer a testable mechanistic hypothesis that toxicity is at least in part the expression of potent and persistent thyroid hormone activity (responses induced by TCDD which qualitatively correspond to those mediated by thyroid

hormones). This mechanistic model sheds light on the diversified effects of TCDD and related compound toxicity and on certain thyroid hormone action.

The effects of TCDD on tadpole growth and development were studied. For reasons of safety and convenience in these preliminary studies, the effects were measured in a nonconventional way through the use of serial photographs. The preliminary finding of earlier and more rapid metamorphosis of tadpoles in the low dose group (0.1ppb in water) is consistent with the proposal that TCDD can function as a thyroid hormone agonist and interact directly with relevant tissue receptors. The fact that some tadpoles in this group never undergo metamorphosis and the frogs that are formed die at a faster rate than controls suggests that the dose response curve for thyroid hormone activity of TCDD is steep and that toxicity is the expression of potent and persistent thyroid hormone activity. Although we made no attempt in this study to measure TCDD levels in tadpole tissue, the persistence of TCDD in animal tissue is well documented. The dose-response pattern seen for toxicity in this study along with the very low concentrations of TCDD required to produce effects are consistent with the potency and persistence of TCDD as a thyroxine agonist. TCDD is also a potent embryotoxic agent in rats and mice exhibiting an extremely steep dose response curve consistent with its effects on tadpole metamorphosis, a somewhat analogous developmental process. Our reproducible results are in contrast to the no-effects results reported by the previous workers who attempted to inject TCDD into the tadpoles.

In preliminary studies, we are examining the utility of monitoring murine stem cell proliferation and differentiation as a model for studying the relationship between thyroid hormones and TCDD toxicity. Combinations of L-T3 and T4 (4:1) at supraphysiological concentrations suppress CFU-GM proliferation similar to TCDD and the effects of either compound are inhibited by a common antagonist, NH₂-TricDD. Furthermore, as previously demonstrated with TCDD-induced cleft palate, either T3 or T4 can potentiate TCDD-induced myelotoxicity. The fact that T3 or T4, alone, had no effects on CFU-GM production at the concentrations tested indicated that combinations of L-T3 and T4 are much more effective in producing thyroid hormone activity than either hormone alone. The use of a 4:1 ratio of T3 to T4 was based upon estimated intracellular concentrations known to occur. There is clearly a stereospecific effect of T3 on the potentiation since D-T3 is less effective in supporting potentiation than L-T3. Our results are consistent with the greater hormonal activity of L-T3 than T4, if T3 is viewed as a potentiator of T4 activity. Thus if TCDD is a T4 agonist, the T3/T4 and T3/TCDD experiments are structurally equivalent results and should produce similar results.

B. Plans for Subsequent Year:

In future studies, we plan to explore the use of the hypothyroid rat animal model to reduce proteins to basal levels and sensitize the animals for parallel studies using dioxin and thyroxine as prototype compounds. In addition to the effects of these compounds on aryl hydrocarbon hydrolase (AHH) activity, a marker enzyme system for dioxin toxicity, we will examine other proteins known to be under

thyroid hormone control. We also plan to use a cDNA probe for the specific mRNA for one or more of these proteins to link these responses to gene expression.

Other collaborative studies will investigate the possible interrelationships between dioxin and iodine which may affect the toxicity caused by dioxin (and related compounds).

C. Publications of past 18 months

Birnbaum, L.S., Weber, H., Harris, M.W., Lamb, J.C., IV and McKinney, J.D.: Toxic interaction of specific PCBs and TCDD: Increased incidence of cleft palate in mice. Toxicol. Appl. Pharmacol. 77: 292-302, 1985.

Lamb, J.C., Harris, M.W., Weber, H., McKinney, J.D., and Birnbaum, L.S.: Potentiation of TCDD induced cleft palate by T₃ in C57 B1/6N (B6) mice. Biol. Reprod. 30: 164, 1984.

Birnbaum, L.S., Harris, M.W., McKinney, J.D., and Lamb, J.C.: Toxic interaction of TCDD with thyroxine. Enhanced incidence of cleft palate in mice. The Pharmacologist 26: 232, 1984.

McKinney, J.D., Chae, K., McConnell, E.E., and Birnbaum, L.S.: Structure-induction versus structure-toxicity relationships for PCBs and related aromatic hydrocarbons. Proceedings of the Conference on Health Effects of PCBs and Related Compounds, Environ. Health Perspect. 60: 57-68, 1985.

McKinney, J.D.: Molecular basis of chemical toxicity in special issue on structure-activity correlations in mechanism studies and predictive toxicology. Environ. Health Perspect. 61: 5-10, 1985.

McKinney, J.D., Fawkes, J., Jordan, S., Chae, K., Oatley, S., Coleman, R.E., and Briner, W.: TCDD as a potent and persistent thyroxine agonist. Mechanistic model for toxicity based on molecular reactivity in special issue on structure activity correlations in mechanism studies and predictive toxicology. Environ. Health Perspect. 61: 41-53, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 80008-11 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthesis of Prostaglandins, Hydroxy-Fatty Acids and Leukotrienes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Prostaglandin Biochemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

3.6

PROFESSIONAL:

2.2

OTHER:

1.4

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Pulmonary tissue produces high amounts of prostaglandins (PG), leukotrienes (LT), and hydroxy fatty acids (HFA) in response to a number of stimuli or pathological states. These lipids have diverse biological activities. The goal of this study is two-fold: first to develop an understanding of factors that control the biosynthesis of these lipids and second to determine their role in the secretory and inflammatory processes of the lung.

We have chosen to study the biosynthesis of these lipids in dog tracheal epithelial cells and to determine their role in control of Cl⁻ and mucus secretion. Dog trachea cells make primarily PGD₂ and a number of LTs. LTC₄, LTB₄ and two unknown LTs are also produced. While Cl⁻ secretion appears to be primarily under control of PGD₂ formation, other data suggest a regulatory role for the LTs. We intend to characterize further the LTs formed and relate them to Cl⁻ secretion.

We are also studying the possible role of these lipids in lymphocyte responses to mitogens and the involvement with protein kinase C.

A. Biosynthesis of Prostaglandins, Hydroxy-Fatty Acids and Leukotrienes

Nature of Problem: Arachidonic acid is metabolized to prostaglandins, thromboxanes, hydroperoxy and hydroxy fatty acids and leukotrienes. These lipids have extremely diverse biological activities and have been implicated in numerous biological processes. Little is known about the biochemistry of arachidonic acid metabolism and even less is known about mechanisms that control arachidonic acid metabolism. Our aim is to develop an understanding of these processes particularly as related to environmental-induced toxicity or disease states. Cystic fibrosis is a pulmonary disease associated with disturbance in water and electrolyte movement and increased mucus secretions. These processes appear to be controlled or modulated by arachidonic acid metabolites. Dog tracheal epithelial cells are model for this disease state. Also the presence of peroxides in tissues alters arachidonic acid metabolism particularly the formation of PGI_2 . The hydroperoxidase of prostaglandin synthase, which reduces peroxides may be a control point for arachidonic acid metabolism.

Objectives: The objectives of this study are: (a) to examine the mechanism and control of biosynthesis, particularly the role of peroxides in this process (b) to characterize the arachidonic acid metabolites by dog tracheal cells and study the relationship to Cl^- and mucus secretion (c) to characterize the arachidonic acid metabolites in isolated cells of potential usefulness in studies of chemical co-oxidation and (d) to study mechanisms involved in prostaglandin biosynthesis (e) to study the possible role of these lipids in cell response to mitogens and characterize the metabolites produced by lymphocytes.

Experimental Approach: Prostaglandin (PG), thromboxane (TX), hydroxy-fatty acid (HFA) and leukotriene (LT) synthetase activities were measured *in vitro* using the microsomal protein from a variety of tissues and organs as an intermediate enzyme source. ^3H or ^{14}C -Arachidonic acid (AA) or prostaglandin or leukotrienes intermediates were incubated at 37°C for various times and under several conditions. After incubation, the PGs and LTs were removed by solvent extraction, separated by high pressure liquid chromatography, and estimated by liquid scintillation techniques. Metabolites were also separated and quantitated by a GC method recently developed. Metabolites were characterized by GC-MS techniques.

Biosynthesis was studied by adding labeled arachidonic acid to cells isolated from rat, dog and rabbit lung. PGs were separated by HPLC. In some experiments cells were maintained in culture for various lengths of time, trypsinized, counted, sonicated, and PGs analyzed. PG biosynthesis was also studied using microsomal fractions. Alveolar macrophages were isolated from rat lung and cultured using standard procedures. Intact dog trachea epithelial cells were isolated and Cl^- secretion measured by electrical potential. Mouse skin cells were isolated and separated into purified cell fractions. Spleen lymphocytes were obtained from mice by disslocation of spleen.

Recent Accomplishments: An HPLC method was developed which permits rapid and nearly complete separation of major arachidonic acid metabolites. The method is based on the use of radiolabeled arachidonic acid as a means of quantitating the metabolites. Leukotrienes are also measured by UV absorbances at 280 nm.

Recently we have developed a GC method for the separation of PGs, LTs and hydroxy fatty acids. These lipids are converted to pentafluorobenzyl oxime derivatives which can be gas chromatographed. The pentafluoro group allows quantitation by the electron capture technique. These derivatives can also be used for mass spectrometric analysis of the metabolite. This method allows us to separate, quantitate and identify metabolites without the use of radioisotopes.

We have characterized arachidonic acid metabolism by dog tracheal cells. These cells make PGD₂, LTC₄ and LTB₄ plus two unidentified metabolites. The time course for the formation of PGD₂ correlates with chloride secretion. Indomethacin inhibits PGD₂ formation and Cl⁻ secretion. PGD₂ added to cells increases chloride ion secretion. Basal level of PGD₂ formation also correlates with basal Cl⁻ secretion. We have now extended our studies to the characterization of AA metabolites produced by human bronchial and nasal epithelial cells. We have observed that human tissue metabolize arachidonic acid to 14,15-LTA₄ rather than 5,6-LTA₄ produced by dog epithelial cells. Moreover, little or no PG's were produced. Cl⁻ secretion was not stimulated by ionophore and not controlled by endogenous prostaglandin formation. Further studies are required to fully characterize the human system.

We have also studied the relationship between phagocytosis of particles and arachidonic acid metabolite biosynthesis in alveolar macrophages. Inhibition of phagocytosis did not inhibit arachidonic acid biosynthesis. Particles binding to sialic acid residues on the macrophage membrane stimulated arachidonic acid release and subsequent oxidation.

The oxidation of arachidonic acid by leukocytes was examined. Metabolism of arachidonic acid was catalyzed by 5- and 15-lipoxygenase enzymes. The major metabolite was LTA₄ but a significant amount of 14,15-LTA₄ was observed. In addition, we observed two additional arachidonic acid metabolites that appear to be catalyzed by cytochrome P450.

We have also investigated the possible role of arachidonic acid metabolism in the mitogenic response of mouse lymphocytes. The addition of a lipoxygenase but not PHS inhibitor reduced the mitogenic response suggesting a role of lipoxygenase in the response. Also the addition of a reducing cofactor for PHS peroxidase significantly inhibited the response. These results suggest a role for hydroperoxides produced by lipoxygenase in cell division.

We have also studied the effect of vitamin K on PGI₂ biosynthesis. The addition of vitamin K₁ to endothelial cells, produced a dose dependent inhibition of PGI₂ formation. Inhibition of biosynthesis could not be demonstrated by *in vitro* subcellular preparations. We determined that vitamin K inhibited phospholipase A₂ preventing the liberation of arachidonic acid. Thus vitamin K inhibited both PGI₂ and PGE₂ productions. The effect of vitamin K appears to be cell specific. No inhibition was observed with macrophages, lymphocytes, platelets but was observed with bovine, porcine or human endothelial and smooth muscle cells.

B. Plan for Subsequent Years

To study arachidonic acid in human bronchial epithelial cells and relate to Cl^- and mucus secretion. We intend also to obtain tissues from cystic fibrosis patients and examine arachidonic acid metabolism. This may give a clue to the disturbance in the metabolism responsible in part for the disease.

To fully characterize the role of vitamin K in PGI biosynthesis and to elucidate mechanism responsible.

To investigate in more detail the involvement of arachidonic acid metabolites in the mitogenic response by lymphocytes and to also investigate these effect in 3-T-3 cells. These studies may lead to a understanding of role of peroxides in protein kinase C activation, and an involvement with phosphatidylinositol metabolism.

To fully characterize the arachidonic acid metabolites produced by human tissue and mouse lymphocytes.

C. Publications of past 18 months

Marnett, L., J., Siedlik, R. H., Ocho, R., Honn, K.V., Das, M. Warnock, R., Tainer, B. and Eling, T.: Mechanism of the stimulation of prostaglandin H synthase and prostacyclin synthase by the anti-thrombotic and antimetastatic agent, Nafazatom: Mole. Pharm. 26: 328-335, 1984

Eling, T. and Ally, A.: Pulmonary biosynthesis and metabolism of prostaglandins and related substances. Environ. Health Perspect. 55: 159-168, 1984.

Henke, D., Kouzan, S. and Eling, T.E.: Analysis of leukotrienes, prostaglandins and other oxygenated metabolites of arachidonic acid by high performance liquid chromatography. Anal. Biochem. 140: 87-94, 1984.

Kouzan, S., Brody, A., Nettesheim, P., Eling, T.E.: Asbestos stimulates the release of arachidonic acid metabolites from alveolar macrophages. Am. Rev. Respir. Dis. In press.

Reed, G., Lasker, J., Eling, T. and Sivarajah, K.: Peroxidative oxidation of bilirubin: Prostaglandins. In press.

Gelen, X., Wu, R., Nettesheim, P., Sivarajah, K. and Eling, T.: Biosynthesis of prostaglandins by isolated and altered airway epithelial cells. Exp. Lung Research. In press.

Boucher, R., McMillan, R., Henke, D., and Eling, T.: Modulation of chloride ion secretion by dog trachea epithelial cells by arachidonic acid metabolites. J. Biol. Chem. In press.

Reed, G., and Eling, T.: Mechanism of inactivation of prostaglandin H synthase and prostacyclin synthase by phenylbutazone. Mole Pharm. 27: 109-114, 1984.

Kouzan, S., Eling, T., and Brody, A.: Binding of iron beads to siliac acid residue on macrophage membrane stimulates arachidonic acid metabolism. Lab. Invest. in press.

Luster, M.I., Tucker, A.N., Hayes, H.T., Pung, O.J., Burka, T., McMillan, R., and Eling, T.: Immunosuppressive effects of benzidine in mice: Evidence of alterations in arachidonic acid metabolism. J. Immunol. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 80035-09 LMB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cooxidation of Xenobiotics by the Prostaglandin Synthetase		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
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	Ronald Mason	Research Chemist LMB NIEHS
	Gregory Reed/Thomas Petry	Staff Fellow LMB NIEHS
	Robert Krauss	Biologist LMB NIEHS
	John Curtis	Chemist LMB NIEHS
	Marc Reilly	Chemist LMB NIEHS
	Jorg Schrieber	Visiting Fellow LMB NIEHS
COOPERATING UNITS:	Dr. Jack Bend, Laboratory of Pharmacology; and Dr. L. Marnett, Wayne State University; Dr. Gupta, Baylor University	Visiting Associate LP NIEHS
LAB/BRANCH Laboratory of Molecular Biophysics		
SECTION Prostaglandin Biochemistry		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.2	2.6	3.6
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The long range goal of this project is to study the oxidation of chemicals to toxic metabolites by <u>prostaglandin synthetase (PHS)</u> and to demonstrate the significance of this system in <u>chemically induced toxicity or carcinogenesis</u>. We have shown that PHS converts both polycyclic hydrocarbons and <u>aromatic amines</u> to mutagens as measured by bacterial tester systems. Other <u>in vitro</u> studies have demonstrated the formation of electrophilic metabolites that react with macromolecules. <u>Benzo(a)pyrene-7,8-diol</u> is metabolized to an <u>anti-diol epoxide</u> by PHS. We have compared PHS and <u>NADPH-dependent metabolism</u> in <u>mouse skin epidermal cells</u>. The aromatic amine carcinogen <u>2-aminofluorene (2-AF)</u> is metabolized to free radical intermediates by PHS. The stable end products are <u>azo-, nitro-fluorene and 2-aminodifluorenylamine</u>. We have studied the formation of phenolic 2-AF adducts and obtained evidence that 2-AF is oxidized to several free radicals or <u>free radical derived products (nitrenium ion)</u>. These radicals may not only be responsible for covalent binding to DNA but also may indeed be the proximate carcinogenic and mutagenic agents. We have also studied the formation of 2-AF DNA adducts catalyzed by PHS. Several unique <u>2-AF-DNA adducts</u> were detected. We have also shown that 2-naphthylamine is <u>oxidized</u> to unique metabolites by PHS and demonstrated a free radical mechanism for the formation of styrene-GSH adducts. Our studies indicate that PHS activates chemicals to ultimate carcinogenic metabolites which may be of importance in the initiation of tumors in extrahepatic tissue. Thus PHS is an enzyme system that, like cytochrome P-450, is important in the metabolism of xenobiotics. </p>		

A. Cooxidation of Xenobiotics by the Prostaglandin Synthetase

Nature of Problem: Substantial evidence supports the hypothesis that metabolism is a prerequisite for the initiation of tumor formation in animal tissue by carcinogens. Polycyclic aromatic hydrocarbons (PAH) and aromatic amines are oxidized to electrophilic metabolites that bind to critical tissue nucleophilic macromolecules, resulting in cell transformation. Understanding the processes of metabolism is therefore important for understanding tumor formation. This may be necessary for the early detection of new environmental carcinogens and the development of inhibitors of carcinogenesis. It is now well established that the metabolism of many chemicals to reactive intermediates is accomplished by the cytochrome P-450 mixed function oxidase system (MFO). During the oxidation of arachidonic acid (AA) to prostaglandins by prostaglandin synthetase (PHS), chemicals are also oxidized. This peroxidatic oxidation can be considered as cooxidation. PHS is ubiquitous and is particularly rich in target organs like the bladder, lung and skin which have low MFO activity. Cooxidation by prostaglandin synthetase may be an additional enzyme system for activation of chemicals.

Objectives: 1) To characterize the metabolites of carcinogens catalyzed by PHS. 2) To study the mechanism(s) of co-oxidation. 3) To relate chemical metabolism by PHS to development of chemical-induced toxicity. 4) To develop biochemical endpoints necessary to assess importance of co-oxidation in vivo.

Experimental Approach: Metabolites are isolated and separated by HPLC and characterized by U.V. spectra, MS and NMR. In some studies direct detection of the radicals by electron spin resonance is employed. Mutagenic metabolites are detected by a modified Ames test. Initial studies are done using ram seminal vesicles microsomes or horseradish peroxidase (HRP) as a source of peroxidase. Studies proceed to using microsomes from target tissue, cells in culture or explants and eventually to whole animals. Our approach is to understand basic mechanism by developing tools that relate metabolism to toxicity, particularly under in vivo condition.

Recent Accomplishments: We have studied the formation of phenolic 2-AF adducts catalyzed by HRP and PHS. Two adducts were found with BHA, one in which the 2-AF nitrogen is linked to para position of BHA and a second in which the amino group of 2-ADFA is linked to para position of BHA. In both cases the reaction is facilitated by leaving of methoxy group. These findings, together with other available evidence, indicates that 2-AF is metabolized by PHS to free radicals which are responsible for the formation of DNA and phenolic adducts. While nitrenium ion formation cannot be excluded, the N-hydroxylamine can be eliminated as a possible intermediate. We have also studied the oxidation of 2-naphthylamine (2-NA) by PHS and HRP. The major metabolites catalyzed by HRP at pH 5 or 7.4 were dimers coupled nitrogen to nitrogen or nitrogen to carbon. At pH 5.0 in the presence of H₂O₂ PHS also formed dimer but at pH 7.4 in presence of arachidonic acid or H₂O₂ the major metabolite was a quinone imine. The reason for the formation of the unique metabolite by PHS is unknown but under investigation.

We have also studied the formation of 2-AF DNA adducts catalyzed by HRP and PHS. The 2AF-DNA adduct is different from the C-8 2-AF adduct produced by cytochrome P450 and N-OH-2AF. This adduct(s), which is very polar, adheres to glass irreversibly. Previous procedures used by most workers in this area would lose this adduct or the adducts would be disposed of during the workup. The structure is not known but there is linkage between C-7 position of 2-AF and an unknown position on guanine.

Preliminary studies indicate that the tryptophan pyrolysis products are oxidized by PHS. Initial studies suggest the try-P-1 and try-P-2 are excellent substrates and are activated to mutagenic metabolites. Further studies are required.

We have also investigated the formation of glutathione conjugates catalyzed by peroxidases. Using styrene as a model we have found evidence for the formation of styrene-glutathione adducts. The mechanism for adduct formation appears to involve a bound thiyl radical that subsequently adds to the double bond of styrene. We have detected by ESR the radical intermediates involved in the reaction. This represents unique mechanism for the formation of GSH adducts.

We have also studied the oxidation of (+)-BP-7,8-diol by mouse skin cell. These isolated cells convert the diol to the anti-diolepoxide. The stereochemistry and unique sensitivity to BHA indicate peroxyl radical oxidation. After pre-treatment of the mice skin with P-450 inducer, the BP-diol was oxidized to syn diolepoxide by subsequently isolated cells.

B. Plans for subsequent year:

1. We will continue to study the epoxidation of BP-7,8-diol in isolated mouse skin cells. Mouse skin is a useful model of BP induced tumor formation. We will focus on the use of intact skin and measure effect of inhibitors on tumor development (in vivo).
2. We will continue to characterize the PHS dependent 2-AF-DNA adducts. Once characterized these adducts will be used to determine if the co-oxidation pathway is operative in bladder cells and in the dog bladder (in vivo). The post labeling method for DNA adducts is being developed in collaboration with Dr. Gupta.
3. We will continue to study the mechanism responsible for β -naphthylamine oxidation by peroxidase and determine the involvement of peroxyl radicals in metabolite formation.
4. We will attempt to study the formation of radicals derived from various chemicals and carcinogens by PHS using intact cells. In only a few cases have free radicals in cells been detected. These studies will be done by Dr. Schrieber in collaboration with Dr. Mason.
5. We will continue to study the formation of GSH conjugates by the free radical mechanism. We will shift our focus to BP-7,8-diol and arachidonic metabolites.

C. Publications of past 18 months

Reed, G., Brooks, E., and Eling, T.: Phenylbutazone dependent epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene. A new mechanism for prostaglandin H synthase-catalyzed oxidations. J. Biol. Chem. 259: 5591-5595, 1984.

Mason, R.P., Chignell, C.F., Eling, T.E., and Mottley, C.: Free radical formation during the peroxidase-catalyzed oxidation of (Bi)sulfite (Hydrated Sulfur Dioxide). Life Chem. Reports Suppl. 2: 55-63, 1984.

Reed, G., Grafstrom, R., Krauss, R., Autrop H., and Eling, T.E.: Prostaglandin H synthase-dependent co-oxidation of (\pm)-7,8-dihydroxy 7,8-dihydrobenzo(a)pyrene in hamster trachea and human bronchus explants. Carcinogenesis 5: 955-960, 1984.

Eling, T., Reed, G., Mason, R.P., and Boyd, J.: Metabolism of carcinogens by prostaglandin H synthase. In H. Thaler-Dao, A. Paulet and R. Paoletti (eds.). Icosanoids and Cancer. Raven Press, 1984, pp. 63-71.

Boyd, J. and Eling, T.: Evidence for a one-electron mechanism of 2-aminofluorene oxidation by prostaglandin H synthase and horseradish peroxidase. J. Biol. Chem. 259: 13885-13896, 1984.

Eling, T., Mason, R. and Sivarajah, K.: The formation and subsequent decay of the aminopyrine cation radical by prostaglandin H synthase. J. Biol. Chem. 260: 1601-1607, 1985.

Krauss, R. and Eling, T.: Arachidonic acid dependent cooxidation: Does it play a role in the activation of chemical carcinogenesis in vivo? Biochem. Pharmac. - Commentary. 33: 3319-3324, 1984.

Krauss, R. and Eling, T.: Formation of unique arylamine/DNA from 2-aminofluorene activated by prostaglandin H synthase. Cancer Res 45: 1680-1686, 1985.

Boyd, G.A., Pagano, D.A., Zeiger, E. and Eling, T.E.: The Prostaglandin H Synthase-Dependent Activation of 2-Aminofluorene To Products Mutagenic To S. typhimurium Strains TA98 and TA98NR. Mutation Res. Lett. In Press.

Vanderslice, R.R., Boyd, J.A., Eling, T.E., and Philpot, R.M.: The Cytochrome P-450 Monooxygenase System of Rabbit Bladder Mucosa: Enzyme Components and Analysis of Isozyme 5-Dependent Metabolism of 2-Aminofluorene. Cancer Res. In Press.

Boyd, J. and Eling, T.E.: Metabolism of Aromatic Amines by Prostaglandin H-synthase. Env. Health. Presp. In Press.

Krauss, R. and Eling, T.E.: Arachidonic acid-dependent, metabolism of carcinogens and toxicants. In L. Marnett and K. Hamm (eds.) Prostaglandins, Leukotrienes and Cancer. Vol. 1 Arachidonic acid metabolism and tumor initiation, Martinus, N. Loff, 1985.

LABORATORY OF PHARMACOLOGY

LABORATORY OF PHARMACOLOGY

Summary Statement

The Laboratory of Pharmacology carries out research to elucidate the relationships between the transformation and translocation of chemicals and toxicity in various target organs and cells of the body. A multidisciplinary approach is used in these investigations. This laboratory provides a central focus at NIEHS for using pharmacological and pharmacokinetic concepts to characterize, in detail, the biochemical and chemical mechanisms by which environmental contaminants exert biological effects. It plans and conducts studies 1) to determine the metabolic basis for selective/specific damage to certain organs and cell types which is characteristic of some toxins, 2) to elucidate the mechanisms whereby hormones or chemicals with hormonal activity imprint tissues for the expression of specific sex-dependent cytochrome P-450s, 3) to determine the role of membrane structure and function in excretion and toxicity of pollutants, and 4) to identify sensitive biochemical, pharmacological, physiological and pathological indicators of target organ/cell toxicity useful for the early detection and prediction of toxicity in experimental animals and humans. The Laboratory of Pharmacology also serves as a focal point within NIEHS, NIH and DHHS for marine and freshwater biomedical research. In this context we are especially interested in possible direct impact on human health by contaminants present in the aquatic environment (including drinking water) and accumulated by aquatic animals. Presently, the Laboratory of Pharmacology contains the Molecular and Comparative Pharmacology and Cell Pharmacology sections.

A. Molecular and Comparative Pharmacology Section (Head: Dr. J.R. Bend): The overall activity of this group can be described as an integrated, multifaceted effort concerned with understanding the role of chemical metabolism, transport and excretion in the mediation of toxicity such as overt tissue damage, or more subtle effects such as carcinogenesis, mutagenesis and teratogenesis.

For many chemicals, the processes of metabolism are means of both activation and inactivation and the relative activities of these pathways/steps, as well as their location in different cells, and parts of cells, are most critical to the particular outcome of exposure to any given chemical. That these processes of metabolic activation and inactivation are themselves often controlled by genetics, as well as being affected by age, sex, disease and environment, further complicate the understanding of their role in the modulation of the biological activity of any chemical in any tissue or animal species or individual of that species at any specific time of exposure.

Major emphasis is currently focused on toxication-detoxication systems, transport and excretory mechanisms, and membrane toxicity.

Another major purpose of this section is to serve as a national focus for

an aquatic pharmacology/toxicology program -- to promote awareness of and use of certain aquatic species and experimental systems for studies, the results of which will give us a better understanding of human disease and contributions of pollution to such disease.

The collaborative efforts of this group demonstrate both its desire to share expertise where possible as well as to make use of the many opportunities for introducing more powerful and new approaches in this research area of chemical metabolism as related to toxicity. This group also interacts closely with the Cell Pharmacology Section of Dr. J.R. Fouts.

Recent Accomplishments:

1. Dr. Bend's laboratory:

- a. Studies with the cytosolic glutathione S-transferases (GS-T) of various tissues of rat and rabbit, with various cells isolated from rabbit lung, and with GS-T purified from both little skate and humans (in collaboration with Dr. Bengt Mannervik, Stockholm University) have shown that stereoselective catalysis with polycyclic arene oxides and alkene oxides is a useful parameter for the functional characterization of these enzymes.
- b. Biochemical and immunochemical studies have shown that cytochrome P-450 forms 2, 5 and 6 and NADPH-cytochrome P-450 reductase are present in smooth muscle of the rabbit aorta. Striated muscle of rabbit leg also contains these monooxygenase components, but their relative distribution between parenchyma and vasculature in this tissue is still unknown.
- c. N- α -methylbenzyl-1-aminobenzotriazole (α -MB) was synthesized and characterized by exact mass spectrometry and NMR. At a concentration of 1 μ M, this compound inactivates 80% of P-450 form 2, 20% of P-450 form 6 and does not affect P-450 form 5 in rabbit lung. Thus, α -MB is the most potent and isozyme-selective suicide inhibitor of rabbit P-450 yet tested.

2. Dr. Negishi's laboratory:

- a. A female-specific cytochrome P-450 isozyme which catalyzes testosterone 16 α -hydroxylation was purified from livers of phenobarbital (PB)-treated 129/J mice. The male and female testosterone 16 α -hydroxylase activities were shown to be immunochemically unrelated P-450 isozymes.
- b. cDNAs encoding the male stereospecific testosterone 16 α -hydroxylase and female stereospecific testosterone 15 α -hydroxylase were cloned and characterized. The cDNA-16 α hybridized only with 2.0 kb mRNA, and there was about ten times more 2.0 kb mRNA in liver of male than in female 129/J mice. Both cDNAs-15 α hybridized only

with 2.1 kb mRNA, and there was about 6 times as much of this mRNA in liver of female versus male 129/J mice.

- c. Female-specific mouse testosterone 16 α -hydroxylase (PB-induced) was shown to share nucleotide sequence homology with cytochromes P-450_{b/e}, which are induced by PB in rat liver.
- d. The deficiency of inducible testosterone 16 α -hydroxylase in liver of female 129/J mice was shown to be inherited as an autosomal recessive trait which is regulated by the Rip locus.

3. Dr. Philpot's laboratory:

- a. A cDNA probe of 1.7 kb for rabbit cytochrome P-450, isozyme 5 was isolated. The rabbit hepatic "library" prepared is now being screened with antibodies to cytochrome P-450, isozyme 2, NADPH-cytochrome P-450 reductase and flavin-containing monooxygenase.
- b. The formation of N-hydroxy-2-aminofluorene, a potent mutagen, from 2-aminofluorene by rabbit flavin-containing monooxygenase was demonstrated.
- c. Induction of the cytochrome P-450 isozyme analogous to that observed in rabbit lung (P-450 isozyme 6) is also observed in lungs of rats, mice, guinea pigs and hamsters following administration of TCDD. In all species the concentration of protein detected with antibodies to rabbit P-450 isozyme 6 or rat P-450_c increases markedly after induction with dioxin.
- d. Placentas from humans exposed to PCBs were also shown to contain a protein that is immunoreactive with antibodies to rabbit cytochrome P-450, isozyme 6. A good correlation between the relative concentration of this protein and the rates of metabolism of 7-ethoxyresorufin and benzo(a)pyrene was observed.

4. Dr. Pritchard's laboratory:

- a. The existence of an Na independent carrier in brush border membranes (BBM) of the rat kidney was demonstrated, and this carrier, in conjunction with the anion exchanger of the basolateral membrane, may mediate the secretory flux of sulfate. The BBM carrier is an anion exchanger and both bicarbonate and chloride are effective counterions. Moreover, the transport is electro-neutral. Kinetic studies and the use of inhibitors have also shown that the secretory component for sulfate of the mammalian kidney is mechanistically analogous to that of the marine teleost.

B. Cell Pharmacology Section (Head: Dr. J.R. Fouts):

This group investigates the localization of drug and pollutant metabolizing enzyme systems in tissues that serve as interfaces with the environment

(e.g., lung, skin and gut) and is also studying factors which affect chemical-metabolizing systems, the development of these systems in the perinatal period, and species differences in these systems. Cell types are isolated from selected tissues and enriched by elutriation and other centrifugation techniques. The contribution of the metabolic systems in individual cells to target organ and cell toxicity is evaluated.

Assay systems are being developed so that pathways of chemical metabolism can be quantitated in single cells. Such procedures will eventually be extended to other systems, including cells in culture. The scientists in this section frequently collaborate with those in the Molecular and Comparative Pharmacology Section.

Another focus of interest is intestinal function and toxicology at the cellular and subcellular levels. A better understanding of the basic biochemistry, physiology and pharmacology of the normal intestine should permit greater appreciation for the important roles of this organ in absorption and metabolism. In addition, this better understanding of normal function may lead to better methods for the detection of dysfunction and toxicity.

Recent Accomplishments:

1. Dr. Fouts' laboratory:

- a. Using immunochemical procedures (Western blotting) cytochrome P-450 forms 2, 5 and 6 and NADPH-cytochrome P-450 reductase were shown to be localized in Clara cells and alveolar type II cells isolated from rabbit lung. Immunoreactive fragments of forms 2 and 5 and reductase were present in the cells, apparently as a result of proteolysis that occurs during isolation of the cells. Similar proteolytic fragments were also found in isolated rabbit hepatocytes.
- b. Human alveolar type II cells were isolated from lung and enriched up to 80% purity. Alveolar macrophages were the major contaminating cell type in these preparations.
- c. Recent results have shown that the microspectrophotometer can be used to measure overall metabolism of benzo(a)pyrene by individual rabbit hepatocytes. Hepatocytes isolated from animals induced with β -naphthoflavone had much higher monooxygenase activities.

2. Dr. Schiller's laboratory:

- a. Adult, male Charles River Fischer rats treated orally with 60 μ g TCDD were shown to undergo changes in lipid metabolism (marked increases in serum triglyceride and cholesterol) that do not occur in 72 hour fasted rats, which lose an equivalent amount (about 10%) of their body weight as do the TCDD-treated animals.

C. Collaborative Efforts

As can be seen from the individual project descriptions, scientists in the Laboratory of Pharmacology are involved in many activities and collaborative research efforts with scientists here as NIEHS and elsewhere.

Examples of collaborative programs outside of NIEHS for the senior scientists are: Dr. Bend with Dr. Bengt Mannervik of the University of Stockholm, and with Dr. Mike Meredith, Vanderbilt University; Dr. Fouts with Dr. Wolfgang Klinger of the University of Jena, Dr. Maggie Coomes, Howard University; Drs. Yankaskas and Van Scott, University of North Carolina and Dr. Bettie Sue Masters, University of Wisconsin at Milwaukee; Dr. Philpot with Dr. Eric Johnson of Scripps Clinic and Research Foundation, Drs. Paul Thomas and Wayne Levin, Hoffmann-LaRoche, and Dr. Lucy Waskell, Veterans Administration, San Francisco; Dr. Pritchard with Drs. Paul Linser and Margaret James, University of Florida, Gainesville and Dr. Gaylen Neufeld, Emporia State University and Dr. Negishi with Dr. J.E. Shively, City of Hope, California.

The collaborative efforts are cited only to show the extensive interactions of this Laboratory with groups outside NIEHS. In addition to these contacts, those with faculty and researchers in the Triangle area are too numerous to document, but add strength to our activities, peer reviews (in terms of seminars, discussions, exchange of students) and opportunities for advice, new techniques, and short courses not only for our staff but for members of the other institutions as well.

D. Personnel

Two senior scientists, and their research programs, transferred from the Laboratory of Pharmacology to other programs within NIEHS during this fiscal year. Dr. Marshall Anderson and the members of his group (Dr. Steven Reynolds, Dr. Felix Romagna, Dr. Steven Belinsky, Ms. Jill Stowers, Ms. Catherine White and Ms. Julie Angerman-Stewart) transferred to the Biochemical Applications Section of the Biometry and Risk Assessment Program, and Dr. Bruce Fowler and his colleagues (Dr. Peter Goering, Dr. Prakash Mistry, Mr. Hayes Brown and Mr. Michael Megginson) transferred to the Systemic Toxicology Branch, Toxicology Research and Testing Program. New arrivals included Dr. David Miller, an Expert with Dr. Pritchard, Dr. Mitsuhide Noshiro, a Visiting Associate with Dr. Negishi, Dr. James Squires, a Guest Worker with Dr. Negishi, Dr. Takeshi Ichikawa, a Visiting Fellow with Dr. Negishi and Dr. Beresford Stock, a Visiting Scientist with Dr. Bend. Other individuals leaving LP this year were Dr. Zahra Parandoosh, a Visiting Fellow with Dr. Philpot and Dr. Ellen Cheung, a Visiting Fellow with Dr. Bend.

E. Other Activities

Dr. J.R. Bend: Adjunct Professor, Interdepartmental Toxicology Program, Department of Entomology, North Carolina State University, Raleigh; Adjunct

Professor, Curriculum in Toxicology, School of Medicine, University of North Carolina; Member, Editorial Advisory Board for Drug Metabolism and Disposition and Board of Editors, Environmental Health Perspectives; Associate Managing Editor (U.S.A.) for Chemico-Biological Interactions; Associate Editor, Reviews in Biochemical Toxicology; Councilor, Section on "Mechanisms of Toxicology", Society of Toxicology; served on graduate student committees at North Carolina State University and University of North Carolina; Member, Steering Committee for Toxicokinetics Section, WHO-sponsored International Program of Chemical Safety; Presented research seminars at University of Toronto and University of British Columbia; Medical Research Council Visiting Professor at University of British Columbia; Invited Speaker at a Symposium on "Problems of Drug-Related Damage to the Respiratory Tract," West Berlin, Germany, and at the Twenty-Sixth Medicinal Chemistry Symposium, State University of New York at Buffalo.

Dr. J.R. Fouts: Adjunct Professor of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill; Adjunct Professor of Toxicology, Department of Entomology, School of Life Sciences, North Carolina State University at Raleigh; Member, Toxicology Advisory Committee, Faculty of Toxicology, North Carolina State University; Associate Editor, Pharmacological Reviews; Editorial Board of Xenobiotica, Pharmacology, and Journal of Toxicology and Environmental Health.

Dr. M. Negishi: Adjunct Associate Professor, School of Veterinary Medicine, North Carolina State University.

Dr. R.M. Philpot: Adjunct Professor, Department of Entomology, North Carolina State University, Raleigh; Member, Toxicology Advisory Committee, North Carolina State University; Associate Managing Editor (U.S.A.) Chemico-Biological Interactions; Associate Editor Reviews in Biochemical Toxicology; Member, Editorial Board Molecular Pharmacology; Member, Executive Committee of the Drug Metabolism Division, American Society for Pharmacology and Experimental Therapeutics.

Dr. John B. Pritchard: Invited Speaker, Third International Symposium on "Responses of Marine Organisms to Pollution," Plymouth, England; Outside Reviewer, FDA Cooperative Agreement applications on Comparative Drug Metabolism in Fish and Other Aquatic Organisms; Presented research seminars at Duke University Marine Laboratory, Duke University Department of Physiology and University of Connecticut.

Dr. C.M. Schiller: Adjunct Associate Professor, Department of Biochemistry and Nutrition, School of Medicine, University of North Carolina, Chapel Hill; Member of the Faculty of the Graduate Curriculum in Toxicology, University of North Carolina, Chapel Hill; Liaison member, U.S.-EPA Toxic Substances Subcommittee, Science Advisory Board, Washington, D.C.; Member, Digestive Diseases Coordinating Committee, Bethesda, MD; Alternate Member, Nutrition Coordinating Committee, Bethesda, MD; Lecturer in graduate-level courses in Biochemical Toxicology at the University of North Carolina,

Chapel Hill; Sponsor of NIH Postdoctoral Fellows in Toxicology Training Program; Interim President, Triangle Chapter Association for Women in Science; Charter Member, International Society for Regulatory Toxicology and Pharmacology; Invited Speaker at the Third International Symposium on Dioxin sponsored by the American Chemical Society; Elected to the North Carolina State Bar; Member, North Carolina Bar Association, Environmental Law Section, and North Carolina Association of Women Attorneys; Recipient of American Society of Biological Chemists Travel Grant to attend the 13th International Congress of Biochemistry, Amsterdam; Invited Speaker on Dioxin at the National Academy of Sciences, BOETH, and Women in Science and Engineering Lecture Series, EPA, Washington, D.C.; Ad hoc Reviewer - RFP, NIHCD; Ad hoc Reviewer NSF; Recipient of Science and Engineering Congressional Fellowship, Sponsor American Chemical Society through the American Association for the Advancement of Science for 1985-1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 70132-06 LP															
PERIOD COVERED October 1, 1984 to September 30, 1985																	
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of Intestinal Metabolism																	
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 40%;">C.M. Schiller</td> <td style="width: 30%;">Research Chemist</td> <td style="width: 10%;">LP</td> <td style="width: 10%;">NIEHS</td> </tr> <tr> <td>Others:</td> <td>M.W. King</td> <td>NIH-Postdoctoral Fellow</td> <td>NRSA</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>D.E. Chapman</td> <td>Toxicologist</td> <td>UNC</td> <td>NIEHS</td> </tr> </table>			PI:	C.M. Schiller	Research Chemist	LP	NIEHS	Others:	M.W. King	NIH-Postdoctoral Fellow	NRSA	NIEHS		D.E. Chapman	Toxicologist	UNC	NIEHS
PI:	C.M. Schiller	Research Chemist	LP	NIEHS													
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	D.E. Chapman	Toxicologist	UNC	NIEHS													
COOPERATING UNITS (if any) Curriculum of Toxicology, University of North Carolina, Chapel Hill, N.C.																	
LAB/BRANCH Laboratory of Pharmacology																	
SECTION Cell Pharmacology																	
INSTITUTE AND LOCATION NIEHS/NIH, Research Triangle Park, North Carolina 27709																	
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Our research focuses on the development and use of animal model systems to study the regulation of gastrointestinal functions. Of particular concern are the regulation of intestinal absorption and metabolism of nutrients, and the alteration of these normally occurring events in response to oral exposure to biologically active environmental toxins. Currently, our investigations involve the systematic examination of the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin) on normal lipid assimilation. The mechanism of the physiologic changes is monitored with a combination of <u>in vitro</u> and <u>in vivo</u> techniques. In particular, our studies include examination of 1) serum lipid, lipoprotein and triglyceride hydrolase, 2) relationship between food deprivation and hyperlipidemia, 3) mortality after chemically induced changes in serum and/or liver lipids, 4) binding and internalization of serum lipoproteins by liver, and 5) epithelial cell membrane proteins and glycoproteins.																	

B. PROJECT DESCRIPTION

REGULATION OF INTESTINAL METABOLISM1. Research Project

Nature of problem: There is an increasing awareness of the gastrointestinal tract as a major metabolic organ system, however, the metabolic regulation mechanisms of its unique processes are not well defined. The absorptive processes are complicated and require specially differentiated epithelial cells. The assimilation of ingested lipid involves the interaction of several organs in order to digest, solubilize, absorb, synthesize and transport the necessary components through the lymph to the organism. Many ingested environmental toxins are lipophilic and, therefore, would be expected to follow the absorptive route of the dietary lipid which may interfere with normal absorption of lipid nutrients and lipid-soluble vitamins. TCDD is a lipophilic toxin known to affect lipid metabolism in humans after accidental exposure. We have observed effects on intestinal lipid uptake and lipoprotein metabolism. Therefore, TCDD is a useful tool for examining perturbations of lipid assimilation in an animal model. In particular, TCDD is a useful probe to the understanding of chylomicra formation, transport and metabolism, and of mesenteric lymph flow. The relationship between lipid absorption and intestinal epithelial cell protein synthesis or plasma membrane serum lipoprotein receptors remains an enigma. TCDD augments the flow of ingested lipid through the intestinal mucosa as enlarged lipoproteins without stimulating intestinal epithelial cell protein synthesis or the volume of lymph flow, thus, it offers a unique opportunity to study the regulation of these two phases of epithelial cell function independently. Since lipid metabolism involves several organs and TCDD is pleiotropic, interpreting changes in serum and liver lipid parameters is complex. Relating changes in lipid levels to toxicity requires examination of the role of these other organs.

Objectives in near term:

The primary objective is to define the effects of sublethal levels of TCDD on lipid absorption and lipoprotein metabolism in an appropriate animal model (adult male Fischer rat).

Experimental approach and scientific justification:

The animal model employed involves administration of a single low dose of TCDD in corn oil by gastric intubation one week prior to experimentation and/or sacrifice. Under these circumstances, there are marginal changes in body weight, nominal differences in feed consumption, and marked changes in serum parameters, hepatic lipid metabolism, lipid uptake and lipoprotein metabolism. Since TCDD exposure augments lipid absorption, collection of the mesenteric lymph provides an ample source of lipoproteins of intestinal

origin for examination and further experimentation. To study lipoprotein lipid metabolism, the chylomicra are radiolabeled with palmitic acid to provide a radiolabeled triglyceride substrate. Perturbation of metabolism by fasting or by injecting metabolite/antimetabolites, such as adenine (Ad)/4-aminopyrazolopyrimidine (4APP), allows for evaluation of the role of each in the metabolic response to TCDD. Serum lipoproteins conjugated with ferritin and incubated with liver slices provide the means to follow the binding and internalization of the serum lipoproteins. Recent advances in lipoprotein binding studies suggested to us a role for intestinal epithelial cell membranes in the altered regulation of lipid after TCDD exposure. These plasma cell membrane proteins were examined to evaluate this possible role.

Recent accomplishments/significance:

Alterations in serum parameters:

Serum composition:

Changes in serum composition were examined in rats one week after exposure to a sublethal dose of TCDD. These changes were examined in groups of control and TCDD-exposed rats that were fasted 16 h or administered an oral dose of corn oil 6 h before sacrifice. In the fasted groups, TCDD exposure increased serum protein, triglyceride, total cholesterol and phospholipid levels as compared to those of the control group, that is, increases of 28, 78, 117, and 64%, respectively. In the groups administered corn oil, TCDD exposure increased serum triglyceride, total cholesterol and phospholipid levels as compared to those of the control group, that is, increases of 125, 72, and 38%, respectively. A 21-d, post-exposure, time-course study indicated statistically significant increases in serum triglyceride and cholesterol 2-d post-exposure, which continued to the 21-d post-exposure time point. The peak values occurred for serum triglyceride at d-7 post-exposure. In contrast, serum glucose decreased significantly by d-2 post-exposure.

Serum lipoproteins

The classes of lipoproteins were isolated by differential density centrifugation and then analyzed for lipid and apoprotein content. In the TCDD-exposed, fasted animals, the triglyceride concentration (mg/ml serum) was increased in the chylomicra (Chylo), very low density lipoproteins (VLDL) and low density lipoproteins (LDL) and the cholesterol concentration (mg/ml serum) was increased in the Chylo and VLDL as compared to the appropriate control concentrations. The corn oil administration increased the lipid concentrations to a greater extent in some of the TCDD-exposed rat serum lipoproteins than in those obtained from the control rats. The triglyceride concentrations were increased in the TCDD-exposed rat Chylo and the cholesterol concentration was increased in all of the TCDD-exposed rat

lipoproteins. It has been determined that 80% of the endogenous serum triglyceride is produced by the liver while the remaining 20% is of intestinal origin. The increases in triglyceride in the fasted, TCDD-exposed rats is probably due to increased synthesis and/or release from the liver and/or decreased metabolism and clearance during circulation. Augmented absorption and resynthesis of lumenal triglyceride by TCDD-exposed rats has already been demonstrated in our laboratory.

Serum apoproteins:

Delipidation of the isolated lipoproteins and separation by SDS-PAGE revealed changes in the apoproteins of corn oil administered TCDD-exposed rats as compared to the controls. In the LDL and high density lipoproteins (HDL) the \uparrow Apo A-I/ \uparrow Apo A-IV ratio increased in the TCDD-exposed animals. In the fasted group, the only change was an increase in Apo A-I in the TCDD-exposed rat serum LDL as compared to that of the control rats.

Serum enzymes:

These alterations in apoprotein composition of the serum lipoproteins may affect their capacity to function as substrates for serum lipoprotein lipase and lecithin:cholesterol acyltransferase as well as their capacity to bind to the lipoprotein receptors for clearance. Our laboratory has shown previously that lymph Chylo and VLDL have altered apoprotein composition (\uparrow Apo A-I/ \uparrow Apo E) and increased size. Reinjection experiments indicated that the clearance time is longer for TCDD-exposed rats injected with TCDD-exposed Chylo. Current studies with artificial substrates (an emulsion of [14 C]-triolein) indicate no difference in post-heparin serum lipoprotein lipase (LPL) in control and TCDD-exposed rats. Specific inhibitors for LPL and for triglyceride hydrolase (liver enzyme) were used to demonstrate the origin of the serum activity which was found to be predominantly LPL.

Relationship between feed deprivation and hyperlipidemia:

Adult, male Charles River Fischer rats (CR/F) given 1/3 of the acute oral LD_{50-30} dose (60 μ g TCDD/kg body wt) gradually lose weight and decrease feed consumption. One week after the single oral dose in corn oil, there is a statistically significant decrease in body wt (about 10%). To compare the effects of TCDD exposure and body wt loss (due solely to feed deprivation) on lipid parameters, groups of TCDD and 72-h fasted rats were examined. Each group lost approximately 10% body wt, but only the TCDD-exposed animals demonstrated marked increases in serum triglyceride and cholesterol. The serum non-esterified fatty acid levels were unaffected by either treatment. The increase in liver lipid and triglyceride levels in both feed-deprived and TCDD-exposed rats was statistically significant ($p < 0.05$), but the TCDD-exposed groups demonstrated a 2-fold increase. These

results indicate that some body wt loss and decreased feed consumption occurs after TCDD exposure, but that the metabolic response, with respect to lipid metabolism, is not that of a normal rat that has lost a similar amount of body wt due solely to fasting. Thus, changes in lipid metabolism should be analyzed independent of feed consumption.

Alterations in lipid parameters and TCDD-induced mortality:

Adult, male CR/F rats were given 2 times the LD₅₀ dose of TCDD to increase the serum and liver lipid concentrations and to induce mortality. In addition, animals were given 4APP, an agent that decreases serum lipids, or Ad, an agent that prevents the formation of fatty liver, to examine the relationship between changes in lipids and TCDD-induced mortality. The principal effect of 4APP on TCDD-induced mortality (325 µg TCDD/kg body wt) was that it shortened the mean time to death without increasing body wt loss. In contrast, Ad stimulated feed consumption and decreased body wt loss, but the mean times to death were similar. Based on these 30-d mortality studies, 4APP, but not Ad, affects the TCDD-induced mortality in CR/F rats. It is not known if some other agent may more effectively prevent TCDD-induced fatty liver formation and affect mortality in TCDD-exposed rats. However, the TCDD-induced sensitivity to 4APP, based on decreased mean time to death, implies that blocking the release and/or synthesis of triglyceride-rich lipoproteins by the liver may play an important role in the TCDD-induced mortality separate from body wt loss. It is possible that only a small proportion of the serum triglyceride was being utilized by the TCDD-exposed rat, and that the 4APP-induced decrease in serum triglyceride affected available energy source below a critical metabolic level and prompted death. In addition, since 4APP reduces the serum triglyceride levels of the TCDD-exposed rat to those of the control, it is likely that the liver is the source of the increase of endogenous serum triglyceride which we have demonstrated to be increased in the serum VLDL, LDL and Chylo.

Changes in the binding and internalization of serum lipoproteins by liver:

An *in vitro* system has been established in our laboratory to examine the binding and internalization of serum LDL and HDL from control and TCDD-exposed rats by livers obtained from control and TCDD-exposed rats. The 1 mm liver slices were maintained in MEM with 5% LP-deficient fetal calf serum to promote the LP receptors before incubation with LDL-ferritin conjugates for 2 h at 4° and for 0.5 h at 37° to examine binding and internalization, respectively. The liver slices were monitored by immunofluorescence with antisera to control and TCDD-exposed rat serum LDL and to ferritin (Ft). Both LDL- and Ft-associated immunofluorescence were found in the same locations on and in hepatocytes along the periphery of the control liver slices. Based on immunofluorescence observations of three replicate experiments, LDL-Ft prepared from control rat serum was bound and internalized by liver to a greater extent than the LDL-Ft from TCDD-exposed rat

serum. Binding, internalization and ultrastructural localization was verified and quantitated by transmission electron microscopy. Although there was no statistically significant difference ($p>0.05$) in the number of vacuoles observed per cell nor in the vacuole diameter, twice as many of the vacuoles in the control liver slices incubated with control serum LDL-Ft contained Ft as did control liver slices incubated with TCDD-exposed LDL-Ft. Current analysis involves similar experiments with TCDD-exposed liver slices. The increased serum triglyceride and cholesterol levels in TCDD-exposed rats may be related to decreased clearance of lipoproteins by the liver. Decreased lipoprotein clearance may result from altered apoprotein composition and/or altered interaction of lipoprotein with the liver lipoprotein receptors. Our data thus far support both possibilities.

Alteration in epithelial cell membrane proteins:

Many of the effects mediated by TCDD have been attributed to alteration of epithelial cell function. A recent preliminary study demonstrated changes in the SDS-PAGE profile of rat liver plasma membrane proteins. Our proposed studies to examine the internalization of LDL-Ft by TCDD-exposed rat liver slices will provide an evaluation of the LDL-liver receptor which is an integral part of the liver plasma membrane. Another recent study suggested that circulating serum lipoproteins may interact with intestinal epithelial cell plasma membranes and, thus, regulate the flow of lipid through these cells. Since we have demonstrated that TCDD enhances lipid absorption independent of increased lymph volume or stimulated protein synthesis, we were interested in possible alterations to the intestinal epithelial cell membranes. We developed a procedure for isolating plasma membranes from intact intestinal epithelial cells with enhanced levels of Na,K-ATPase from both control and TCDD-exposed rats. SDS-PAGE analysis indicated that the TCDD-exposed membrane profile is altered both for proteins and glycoproteins. Antibodies raised for the control and TCDD-exposed rat cell plasma membrane proteins allow distinguishing the integrity of these membranes. The profile differences suggest that the structural integrity of the epithelial cell membranes is altered and may result in the inability to respond to the increase in circulating serum lipoproteins.

2. Plans for Future

Alterations in lipid metabolism:

The expressed interest in the possible role of altered lipid metabolism in the toxicity of TCDD has led to the emphasis of the increased serum and liver triglyceride levels. The mortality studies indicate that the increased serum triglyceride may provide a critical source of metabolic energy to the organism which when decreased is lethal. This observation emphasizes the importance of the metabolism of the serum lipoprotein triglyceride, lipoprotein binding to the lipoprotein receptor and the

internalization of the lipoprotein by the liver. Thus, ongoing experiments focus on the metabolism of lipoproteins obtained from lymph and serum as substrates for serum enzymes and liver receptors. To quantitate binding differences (non-specific and specific binding), LDL prepared from the serum of control and possibly of TCDD-exposed rats will be iodinated with ^{125}I and incubated with the liver slices in culture. Plasma membranes prepared from livers of control and TCDD-exposed rats will be incubated with ^{125}I -LDL. The SDS-PAGE profile of these complexes will reflect any changes in receptor molecular wt and composition. In addition, it is of interest to discern whether ethynyl estradiol, an agent that induces hepatic lipoprotein receptors, can improve lipoprotein removal and affect serum lipid levels of TCDD-exposed rats.

The serum and liver triglyceride levels in the normal rat vary with strain but are carefully regulated and involve the interactions of the liver, intestines and adipose tissues. After TCDD-exposure, both serum and liver triglyceride levels increase. Since the endogenous serum triglyceride is predominantly of liver origin, it is of interest to determine the source of imbalance in the liver. A priori, there are four general mechanisms by which TCDD might increase the liver triglyceride concentration: 1) through increased triglyceride synthesis, 2) through decreased fatty acid oxidation, 3) through increased uptake of serum triglyceride or 4) through decreased release of lipoprotein triglyceride into the circulation. The effect of TCDD on each of these processes will be studied. Given the pleiotropic nature of TCDD, it is likely that more than one of these processes will be altered.

3. Publications (past 18 months)

Melnick, R.L., and Schiller, C.M.: Effect of phthalate esters on energy coupling and succinate oxidation by rat liver mitochondria. Toxicology 34: 13-27, 1985.

Walden, R., and Schiller, C.M.: Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in four (sub)strains of adult male rats. Toxicol. Appl. Pharmacol. 77: 490-495, 1985.

Chapman, D.E., and Schiller, C.M.: Dose-related effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in C57BL/6J and DBA/2J mice. Toxicol. Appl. Pharmacol. 78: 147-157, 1985.

Schiller, C.M., Chapman, D.E., and Shoaf, C.R.: Alterations of intestinal function by chemical exposure: Animal models. In Schiller, C.M. (Ed.): Intestinal Toxicology. New York, Raven Press, Inc., 1984, pp. 133-144.

Schiller, C.M., Walden, R., Chapman, D.E., and Shoaf, C.R.: Metabolic impairment associated with a single, low, oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Fischer rats: Altered lipid assimilation. In Poland, A., and Kimbrough, E.D. (Eds.): Biological Mechanisms of Dioxin Action. New York, Cold Spring Harbor Laboratory, Banbury Report 18, 1984, pp. 319-331.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 80001-13 LP
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Microsomal Mixed-Function Oxidase Systems: Specificity and Function		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	R.M. Philpot	Research Chemist LP NIEHS
Others:	B.A. Domin	Staff Fellow LP NIEHS
	R.R. Vanderslice	Graduate Student LP NIEHS
	Z. Parandoosh	Visiting Fellow LP NIEHS
	G. Carver	Biologist LP NIEHS
	R. Tynes	Guest Worker LP NIEHS
	R. Gasser	Guest Worker LP NIEHS
COOPERATING UNITS (if any) Department of Pharmacology, Scripps Clinic and Research Foundation, LaJolla, CA; Department of Biochemistry, University of Michigan, Ann Arbor, MI		
LAB/BRANCH Laboratory of Pharmacology		
SECTION Molecular and Comparative Pharmacology		
INSTITUTE AND LOCATION NIEHS/NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
5.5	4.0	1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Rabbit cytochrome P-450, isozyme 5, is highly active in the metabolism of some aromatic amines to mutagenic products. This isozyme, which we initially purified from rabbit lung, can be detected by immunochemical methods in several other tissues including liver and bladder. Following treatment of rabbits with phenobarbital, the hepatic concentration of protein detected by antibodies to isozyme 5 increases about 10-fold; however, no increase in this protein is noted in the lung. Thus, the response of isozyme 5 to phenobarbital is the same as that of isozyme 2 although these two forms of cytochrome P-450 do not appear to be immunochemically or functionally related. We have now purified isozyme 5 from the livers of rabbits treated with phenobarbital and compared it with isozyme 5 from lung. The proteins from liver and lung have been digested with several proteolytic enzymes. No differences are noted when the peptides formed are compared by SDS-gel electrophoresis and "Western blotting". The proteins from liver and lung are also the same with respect to UV-vis spectra, the sequence of the first 20 N-terminal amino acids, and their catalytic activities with 2-aminofluorene. In contrast to isozyme 5 of cytochrome P-450, the flavin-containing monooxygenases of rabbit liver and lung are different enzymes. The relative contributions of cytochrome P-450, isozymes 4 and 5, and the flavin-containing monooxygenase to the metabolism of 2-aminofluorene in rabbit liver, lung and bladder are now being investigated.		

B. PROJECT DESCRIPTION

MICROSOMAL MIXED-FUNCTION OXIDASE SYSTEMS: SPECIFICITY AND FUNCTION1. Research Project

Nature of problem: Cytochrome P-450 monooxygenase systems (P-450 systems) metabolize a multitude of endogenous and exogenous chemicals. As a result, P-450 systems are involved in a myriad of vital metabolic processes associated with the biotransformation and elimination of xenobiotics and the synthesis and catabolism of biochemicals. Unfortunately, cytochrome P-450-mediated metabolism also leads to the formation of some products that react with cellular macromolecules. A number of carcinogenic, mutagenic, and other toxic metabolites are formed from relatively inert compounds by this process.

Cytochrome P-450 consists of a number of isozymes with different substrate specificities. Therefore, the metabolic capabilities of individual P-450 systems are, for the most part, determined by the types and concentrations of the isozymes present. Among other factors, specific isozyme profiles depend upon species, age, tissue, cell type, and exposure of animals to various exogenous chemicals.

Objectives in near term:

It can be concluded that isozymes 2 and 5 from rabbit lung are very similar to the corresponding hepatic enzymes. However, identity has not yet been established. Microheterogeneity has been observed with several forms of cytochrome P-450 from liver, and it is possible that isozymes from various tissues are composed of different populations of highly similar, but not identical, forms. One approach to this problem involves the use of specific cDNA probes and eventual sequencing of nucleic acids. Given the difficulties encountered in working with P-450 protein, the molecular biological approach is warranted. This approach will also allow us to further investigate mechanisms responsible for the marked differences between tissues in their responses to inducers and repressors. cDNA probes for cytochrome P-450 isozymes 2 and 5 and for the flavin-containing monooxygenases will be isolated. These probes, along with monoclonal antibodies, will be incorporated into our studies of monooxygenase systems of different tissues. The roles of isozymes 4 and 5 and the flavin-containing monooxygenase in the metabolism of aromatic amines will be further investigated.

Experimental design:

The lambda GT-11 system and polysomal mRNA from livers of rabbits treated with phenobarbital will be used for the development of cDNA probes. All screening will be done on the basis of protein expression as determined by reactivity with specific antibodies. Hybrid-select in vitro translation

will be used for positive probe identification. Initially, the probes will be used for the determination of mRNA levels in tissues of untreated rabbits and rabbits treated with phenobarbital or TCDD. These results will be compared with those for protein expression, determined by "Western blotting" and activity expression, determined by antibody inhibition. Experiments will be designed with the intent of associating changes in substrate specificity with specific alterations in the synthesis of mRNA and protein and in the extent to which activity is expressed.

Recent accomplishments:

Cytochrome P-450, isozyme 5, has been purified from livers of rabbits treated with phenobarbital. Purification was accomplished by a combination of DEAE-sepharose, DEAE-cellulose, CM-sepharose, hydroxylapatite, and phenyl-sepharose column chromatography. Removal of isozyme 6, whose chromatographic properties are similar to those of isozyme 5, was monitored immunochemically. Similar methods were used for the detection and removal of protein having the same monomeric molecular weight as isozyme 5. With these methods, hepatic isozyme 5 was purified to homogeneity as determined by SDS-gel electrophoresis and "silver-staining". Peptides of liver and lung isozyme 5 formed by digestion with papain, α -chymotrypsin, or *Staph. aureus* were compared by SDS-gel electrophoresis, "silver-staining", and "Western blotting". Minor differences observed with "silver-staining" could not be detected by "Western blotting". Other methods of comparison (spectra, N-terminal sequences, and activities) have also shown no differences. In order to extend our work on isozyme 5, a cDNA probe for this form of cytochrome P-450 has been isolated. The probe was identified and selected by analyzing replicates of cultures on nitrocellulose paper with specific antibodies. A cDNA of about 1.7 kb was obtained with these methods. A hepatic "library" is now being screened with antibodies to cytochrome P-450, isozyme 2, cytochrome P-450 reductase, and the flavin-containing monooxygenase.

The flavin-containing monooxygenases from rabbit liver and lung have been found to differ with respect to their abilities to metabolize straight-chain primary amines. Compounds, such as N-octylamine, which are not metabolized by the hepatic enzyme from rabbit, pig, or mouse, are metabolized by the rabbit pulmonary enzyme. Incubation of N-octylamine with the pulmonary enzymes results in increased utilization of NADPH and the formation of hydrogen peroxide and a product that reacts with a reagent specific for N-hydroxides. The rabbit flavin-containing monooxygenase is also active with 2-aminofluorene from which N-hydroxy-2-aminofluorene is formed. This product has been identified by HPLC.

The metabolism of 2-aminofluorene in microsomal preparations from rabbit lung, liver and bladder is catalyzed primarily by cytochrome P-450, isozyme 5. The involvement of isozyme 4 and the flavin-containing monooxygenase

remain to be determined. The products formed from 2-aminofluorene by isozyme 5 are N-hydroxy-2-aminofluorene, 2-nitrosofluorene, 2,2'-bis-azoxyfluorene, and a ring-hydroxylated metabolite. The primary enzymatic reaction is N-hydroxylation. The nitroso and azoxy products are formed by non-enzymatic breakdown of the N-hydroxy-2-aminofluorene. The ring-hydroxylated metabolite can also be formed non-enzymatically, but the extent that this contributes to total product formation is not clear. Microsomal preparations from rabbit bladder are more active than those from rabbit liver in the metabolism of 2-aminofluorene.

2. Plans for Future

Techniques of molecular biology will be integrated with those of immunochemistry for a more detailed comparison of isozymes 2 and 5 from rabbit liver and lung. A final comparison of the enzymes from the two tissues depends upon a determination of the primary sequences. The induction and repression of isozymes 2 and 5 will also be studied with respect to synthesis of mRNA and protein and expression of activities.

The tissue and species distribution of isozyme 5 will be investigated with monoclonal antibodies. These antibodies will be screened for use in inhibition and blotting experiments. The distribution of isozyme 4 and the flavin-containing monooxygenases will also be determined. Specific inhibitors and antibodies will be used for an analysis of the contribution of each enzyme to the metabolism of 2-aminofluorene. Because major differences in K_m s are expected, these experiments will be done with a wide range of substrate concentrations.

3. Publications (past 18 months)

Philpot, R.M. and Smith, B.R.: The role of cytochrome P-450 and related enzymes in the pulmonary metabolism of xenobiotics. Environ. Health Perspect. 55: 359-367, 1984.

Philpot, R.M., Domin, B.A., Devereux, T.R., Harris, C., Anderson, M.W., Fouts, J.R., and Bend, J.R.: Cytochrome P-450-dependent monooxygenase systems of the lung: Relationships to pulmonary toxicity. In Boobis, A.R., Caldwell, J., DeMatteis, F., and Elcombe, C.R. (Eds.): Microsomes and Drug Oxidations. Proceedings of the 6th International Symposium. London, Taylor and Francis, 1985, pp. 248-255.

Aune, T., Vanderslice, R.R., Croft, J.E., Dybing, E., Bend, J.R., and Philpot, R.M.: Deacetylation to 2-aminofluorene as a major initial reaction in the microsomal metabolism of 2-acetylaminofluorene to mutagenic products in preparations from rabbit lung and liver. Cancer Res., in press, 1985.

Vanderslice, R.R., Boyd, J.A., Eling, T.E., and Philpot, R.M.: The cytochrome P-450 monooxygenase system of rabbit bladder mucosa: Enzyme components and isozyme 5-dependent metabolism of 2-aminofluorene. Cancer Res., in press, 1985.

Serabjit-Singh, C.J., Bend, J.R., and Philpot, R.M.: Cytochrome P-450 monooxygenase system: Localization in smooth muscle of rabbit aorta. Mol. Pharmacol., in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 80007-14 LP																					
PERIOD COVERED October 1, 1984 to September 30, 1985																							
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Conjugation and Oxidation Pathways for Xenobiotic Metabolism																							
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 50%;">PI: John R. Bend</td> <td style="width: 30%;">Chief</td> <td style="width: 20%;">LP NIEHS</td> </tr> <tr> <td>Others: E. Cheung/J. Horton</td> <td>Visiting Fellows</td> <td>LP NIEHS</td> </tr> <tr> <td>D. Brier/C. Serabjit-Singh</td> <td>Chemists</td> <td>LP NIEHS</td> </tr> <tr> <td>M. Coughtrie</td> <td>Guest Worker</td> <td>LP NIEHS</td> </tr> <tr> <td>L. Dostal</td> <td>NIH-Postdoctoral Fellow</td> <td>LP NIEHS</td> </tr> <tr> <td>B. Stock</td> <td>Visiting Scientist</td> <td>LP NIEHS</td> </tr> <tr> <td>T. Eling</td> <td>Research Chemist</td> <td>LMB NIEHS</td> </tr> </table>			PI: John R. Bend	Chief	LP NIEHS	Others: E. Cheung/J. Horton	Visiting Fellows	LP NIEHS	D. Brier/C. Serabjit-Singh	Chemists	LP NIEHS	M. Coughtrie	Guest Worker	LP NIEHS	L. Dostal	NIH-Postdoctoral Fellow	LP NIEHS	B. Stock	Visiting Scientist	LP NIEHS	T. Eling	Research Chemist	LMB NIEHS
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COOPERATING UNITS (if any) Arrhenius Laboratory of Biochemistry, Stockholm University; Laboratory of Molecular Biophysics, NIEHS; Department of Biochemistry, University of Dundee, Scotland; Department of Biochemistry, Vanderbilt University																							
LAB/BRANCH Laboratory of Pharmacology																							
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INSTITUTE AND LOCATION NIEHS/NIH, Research Triangle Park, North Carolina 27709																							
TOTAL MAN-YEARS 5.6	PROFESSIONAL 2.6	OTHER 3.0																					
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																							
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Epoxides are frequently formed as metabolites of unsaturated hydrocarbons by cytochrome P-450-dependent monooxygenase activity. Many arene and alkene oxides are known to react covalently with macromolecules and to transform cells <u>in vitro</u>, suggesting they are ultimate carcinogens, mutagens or cytotoxins. We are studying various aspects of the enzymatic formation and metabolism of epoxides and of glycols and phenols which are products of subsequent epoxide biotransformation, in relationship to cell-selective and organ-selective toxicity of compounds metabolized to epoxides by both <u>hepatic</u> and <u>extrahepatic</u> tissues. Particular attention is given to the <u>respiratory tract</u> because this is a common site for pollutant-mediated damage. We are currently investigating <u>stereochemical</u> aspects of <u>styrene</u> oxidation, stereochemical and kinetic aspects of the biotransformation of model alkene (styrene 7,8-oxide) and polycyclic arene (benzo(a)pyrene 4,5-oxide) oxide substrates by cytosolic and purified <u>glutathione transferases</u> of diverse origin, the distribution and characteristics of components of the cytochrome P-450-containing monooxygenase system in <u>vascular endothelium</u>, the status of the tripeptide <u>glutathione</u>, which is important in detoxication of electrophilic metabolites, in perfused <u>lung</u>, <u>Clara cells</u>, <u>alveolar type II cells</u>, and <u>alveolar macrophages</u> isolated from <u>rabbit lung</u> before and after exposure to electrophilic metabolites or conditions of oxidant stress (e.g., exposure to <u>paraquat</u>), and the biochemical and immunochemical properties of pulmonary and renal UDP-glucuronosyltransferases.</p>																							

B. PROJECT DESCRIPTION

CONJUGATION AND OXIDATION PATHWAYS FOR XENOBIOTIC METABOLISM1. Research Project

Nature of problem: Unsaturated hydrocarbons, such as the polycyclic aromatic hydrocarbons and alkylated benzenes, occur as common environmental pollutants. Potentially toxic electrophilic compounds, including epoxides are formed as in vivo and in vitro metabolites of hydrocarbons. We are investigating the chemical/biochemical characteristics of the enzymes responsible for formation and subsequent biotransformation of toxic hydrocarbon metabolites in liver and extrahepatic tissues. Attention is focused on the cytochrome P-450-dependent monooxygenase system, the glutathione transferases and UDP-glucuronosyltransferases with regard to expression of target organ and/or cell toxicity.

Objectives in near term:

1. To investigate the stereoselectivity of cytosolic and purified glutathione transferases with selected alkene and arene oxide substrates and to purify and characterize biochemically and immunochemically selected isozymes because of unique stereochemical properties. For example, some of the rabbit liver and lung glutathione transferases which are apparently stereospecific for reaction of GS^- with an S-configured oxirane carbon atom of polycyclic arene oxides will be characterized. (The marked differences we have observed in stereoselectivity of the glutathione transferases with epoxide substrates suggest that this will be a useful parameter for the functional characterization of these dimeric proteins and their subunits. This hypothesis is currently being tested.)
2. To determine the mechanism for the formation of styrene-glutathione adducts in incubation mixtures containing peroxidative enzymes such as prostaglandin H synthase and horseradish peroxidase. Since the two major glutathione (GSH) adducts that result from the reaction of styrene 7,8-oxide and GSH are not formed in these reaction mixtures, it seems obvious that styrene oxide is not an intermediate in the reaction. (We are currently testing the hypothesis that a sulfur free radical ($\text{GS}\cdot$) is formed which reacts with styrene at the terminal carbon atom.)
3. To identify by immunochemistry and immunocytochemistry the isozymes of cytochrome P-450 present in aorta, heart and smooth muscle of rabbit and to accurately determine the monooxygenase activities associated with these forms of P-450 in these tissues. (The vascular endothelium represents the largest surface area in intimate contact with circulating chemicals. We are investigating the ability of this tissue to oxidatively metabolize exogenous compounds.)

4. To study the GSH status (i.e., biosynthesis, degradation, release) in Clara cells, alveolar type II cells and alveolar macrophages freshly isolated from rabbit lung before and after exposure to chemicals that are known to alter GSH status (e.g., paraquat, styrene 7,8-oxide). (Reaction with GSH is an important detoxication reaction for many chemical toxins. We are investigating relationships between GSH status and xenobiotic-mediated target cell toxicity in lung).

5. To study the ability of populations of tracheal cells to metabolize selected xenobiotics when freshly isolated from untreated vs. TCDD- or β -NF-induced rabbits, to determine the relative amounts of P-450 isozymes 2, 5 and 6 present in tracheal microsomes from untreated and induced rabbits, and to identify by immunochemistry and immunocytochemistry the P-450 isozymes present in various portions/cells of the trachea.

6. To compare biochemically and immunochemically the UDP-glucuronosyltransferase enzymes present in rat lung and kidney to those of liver. (Preliminary experiments suggest that both the lung and kidney contain some different forms of UDP-glucuronosyltransferase than are present in liver. We intend to purify and characterize some of these "unique" forms and to eventually study the differential regulation of these enzymes in various tissues and cells.)

Experimental approach and scientific justification:

General:

An integrated experimental approach is used, and studies are conducted with purified enzymes and antibodies elicited against these proteins, subcellular fractions of various tissues, freshly isolated pulmonary cells, isolated perfused organs and intact animals. With this combination of procedures we are generally able to pursue a problem to a logical conclusion. Investigations in systems with intact cellular structure are especially important for those chemicals whose activation/deactivation depend on sequential enzymatic reactions localized in different cellular organelles or in different cells/tissues. Highly purified, radiolabeled substrates are used in most experiments. Metabolite isolation, identification and quantitation are most frequently accomplished using high performance liquid chromatography (HPLC) and scintillation counting. Detailed characterization (^{13}C -NMR, NMR, mass spectroscopy, etc.) and synthesis of metabolites when required are routinely accomplished in collaboration with scientists in the Laboratory of Molecular Biophysics. The Western blotting technique of Towbin et al. with subsequent immunochemical staining is used both for identification (by monomeric molecular weight) and quantitation (by densitometry) of cytochrome P-450, glutathione transferase, and UDP-glucuronosyltransferase isozymes. Cytochemistry of tissue sections by immunofluorescence and immunoperoxidase

staining is used to determine the cellular localization of specific forms of cytochrome P-450, glutathione transferase or glucuronosyltransferase.

This combination of chemical, biochemical, immunochemical and pharmacological approaches allows us to investigate the chemical and biochemical mechanisms involved in xenobiotic activation and detoxication at the organ, cell and molecular levels.

Recent accomplishments:

1. Stereoselectivity of glutathione transferases (GT). We have shown that cytosolic GT of rat liver, lung, testis and heart are stereoselective for thiol reaction with R-configured oxirane carbon atoms of (\pm)-benzo(a)-pyrene 4,5-oxide (BPO), (\pm)-benz(a)anthracene 5,6-oxide (BAO) and pyrene 4,5-oxide (PO). In contrast, kidney and spleen cytosol were highly stereoselective for reaction of GSH with S-configured carbon atoms of these three arene oxides. The enantioselectivity of GS-T with racemic BPO as substrate also varied substantially from tissue to tissue. Liver, kidney, spleen and intestine preferentially catalyzed the reaction with the (4R,5S)-BPO enantiomer (2.8- to 5.1-fold excess) but testis, lung and heart showed little or no enantioselectivity. Cytosolic GT of rabbit liver, lung, kidney, testis and intestine were found to be stereoselective for the S-configured epoxide carbon atoms of BPO, BAO and PO; in fact, rabbit liver was almost stereospecific for thiol reaction at this site with all three substrates (R/S = 0.03, 0.01 and 0.03, respectively). This investigation was extended to cytosolic fractions prepared from hepatocytes, alveolar type II cells, Clara cells and alveolar macrophages isolated from rabbits. Both the specific activity and the stereoselectivity of the GT in the two pulmonary epithelial cell types and the hepatocytes were very similar to those observed in whole lung and whole liver cytosol, respectively. On the other hand, specific activity was considerably lower in the alveolar macrophage and the stereoselectivity was opposite to that observed in lung cytosol.

Collaborative studies with Dr. Bengt Mannervik, Stockholm University, have shown that the stereoselectivity of three biochemically and immunochemically distinct human GT (placenta, π ; liver, μ ; and liver, α - ξ) varies markedly with BPO or PO as substrate. Thus, the π enzyme is almost stereospecific for thiol reaction with an S-configured oxirane carbon atom, the μ enzyme is almost stereospecific for reaction with an R-configured carbon atom and the α - ξ enzymes are intermediate in stereoselectivity, showing a slight preference for an R-configured carbon atom (R/S = 2.1 for BPO and 1.6 for PO). In concert, these data demonstrate that stereoselectivity of GT with polycyclic arene oxides is a useful parameter for their functional characterization, and that these compounds serve as excellent probes for studies of the topography of the substrate binding site.

2. In a collaborative project with Drs. Eling and Mason, LMB, we have shown that two, presumably diastereomeric, styrene-glutathione adducts are formed from styrene and GSH in the presence of prostaglandin H synthase, the first enzyme of the prostaglandin cascade. The mechanism of adduct formation is by reaction of the GSH radical ($GS\cdot$) with styrene. Preliminary ESR studies have shown that at least three radical species can be spin trapped during the course of reactions containing glutathione, styrene, aminopyrine and horseradish peroxidase; these include $GS\cdot$, a carbon centered radical of styrene and the aminopyrine radical.

3. Cytochrome P-450 isozymes in aorta and heart of the rabbit: Western blots of heart and aorta microsomal fractions demonstrated the presence of immunoreactive proteins of monomeric molecular weight equivalent to those of P-450 forms 2, 5, and 6 and NADPH-cytochrome P-450 reductase. Treatment with TCDD resulted in the induction of form 6, while levels of the other enzymes were unchanged. Induction of 7-ethoxyresorufin O-deethylase was observed in microsomes from aorta or heart of rabbits treated with TCDD. The content of these proteins in smooth muscle of the aorta is significant (i.e., immunoreactive monooxygenase elements do not appear to be concentrated in the intima as suggested by the cytochemical data of others). Both immunoreactive proteins and NADPH-cytochrome c reductase activity were detected in microsomes from aortas which had been stripped of endothelium, as confirmed by microscopy. No benzphetamine demethylation activity (<100 pmol/min/mg protein) was observed, however, consistent with the minimal content of P-450, form 2 indicated by Western blotting. In addition to the heart, striated muscle from the leg contains monooxygenase components but it is unclear what portion is due to the vasculature as compared to the parenchyma.

The vascular endothelium represents the largest surface area in intimate contact with circulating chemicals, both endogenous and exogenous. The contribution of this tissue, if metabolically active, would be important not only because of size but also because of proximity to all other tissues. Preliminary experiments to determine the immunoreactivity of cultured bovine endothelial cells were negative and due to the poor yield from plating porcine endothelial cells, we have not as yet determined whether these cells contain monooxygenase components. Clearly, the nature, amount and activity of metabolic activation/detoxication enzymes, such as P-450, must be determined if a correlation between vascular metabolism of chemicals and disease states is to be established. Moreover, delineation of the distribution of these enzymes in functionally diverse tissues may provide insight to their physiological roles.

4. Glutathione (GSH) status of lung: The ability of the ^{35}S -sulfur amino acids L-cysteine, L-methionine and L-cystine to support GSH biosynthesis was studied in freshly isolated preparations of rabbit type II cells, Clara cells and alveolar macrophages. Preliminary studies demonstrated a high rate of GSH synthesis in macrophages incubated with L-cysteine.

Lower rates of synthesis were observed with this precursor in type II and Clara cells, and more GSH was detected extracellularly. L-Methionine did not support GSH biosynthesis in any of the cell types although this amino acid is taken up rapidly by the cells. Perfusion of rabbit lung with L-cysteine (1 mM) allowed resynthesis of GSH, with greater rates of synthesis occurring in the lungs with lowest (diethyl maleate depletion) GSH content.

Paraquat (PQ; 10 μ M), a lung-specific toxin in several species, was taken up by cell populations enriched in rabbit alveolar type II and Clara cells (intracellular concentration was about 10-fold higher than media concentration after 60 min). Uptake was inhibited by equimolar concentrations of the diamine, putrescine and by a combination of KCN and iodoacetate, suggesting that PQ uptake is occurring by the energy-dependent process that functions for endogenous di- and polyamines. The reduction of PQ (1 mM) in enriched populations of lung cells was investigated by ESR (in collaboration with Ron Mason, LMB). Whereas the ESR signal of the PQ anion radical increased with time with intact type II and Clara cell preparations, a signal was only observed in sonicated macrophages. Our data suggest that the major reason for not finding an ESR signal in intact macrophages is the absence of the energy-dependent uptake system in these cells because the magnitude of the ESR signal was decreased in type II cells in the presence of putrescine (10 mM). The fact that addition of an antibody to NADPHcytochrome P-450 reductase did not affect the rate of appearance or magnitude of the PQ radical signal in whole cells provides evidence that the radical is being formed intracellularly.

5. The trachea as a site of cytochrome P-450-dependent monooxygenase activity. P-450-dependent monooxygenase activity was demonstrated in tracheal microsomes of the rabbit and in a mixed population of tracheal cells prepared from this species with benzo(a)pyrene (BP), 7-ethoxycoumarin (7-EC) and 7-ethoxyresorufin (7-ERF) as substrates. Microsomal activities were increased with all three substrates following pretreatment with β -naphthoflavone (β -NF) or TCDD. Although β -NF had no effect on the overall rate of BP metabolism in sonicated tracheal cells (isolated from rabbits treated with β -NF), there were marked increases in the amount of oxidation occurring on the benzo ring of BP (i.e., positions 7, 8 and 9).

The cilia of the upper but not lower airways exhibited immunofluorescence or immunoperoxidase staining with the antibodies against the major pulmonary monooxygenase components (P-450 forms 2, 5 and 6 and NADPH-cytochrome P-450 reductase). This staining is not an artifact of fixation: the lumen of native unwashed tracheas was shown to contain these epitopes upon immunostaining a nitrocellulose contact blot. The monomeric molecular weights (Western blot analysis) of the immunoreactive proteins obtained by tracheal lavage were the same as the homologous antigens, and the reactivity was shown to be associated with membranes in the lavage. The source of these membranes in the trachea is still unknown.

6. Extrahepatic UDP-glucuronosyltransferase (UDPGT). AN HPLC system was developed which follows product formation of the ^{14}C -labeled glucuronides of a wide range of substrate aglycones. The sensitivity and broad applicability of this system is currently being used to investigate differences in the substrate selectivities of pulmonary and renal UDPGTs from those of liver. Using antibody raised against purified rat liver UDPGT, we have shown that hepatic and pulmonary GT activity (1-naphthol as substrate) can be inhibited, but that the antibody did not cross react well with pulmonary or renal UDPGTs on Western blots. Consequently, we have purified GTs from rat renal microsomes. Based on Western blotting and SDS-polyacrylamide gel electrophoresis analyses, two forms of renal UDPGT copurify with monomeric molecular weights of 56,500 and 59,000 Daltons.

2. Plans for Future

1. Stereoselectivity of glutathione transferases. GT will be purified from rabbit liver and lung because cytosol from these sources shows a marked stereoselectivity for S-configured oxirane carbons. Purified proteins will be characterized and will be used for the preparation of antibodies (polyclonal initially, but monoclonal eventually). In these studies particular attention will be given to subunit composition (i.e., homodimers vs. heterodimers) and to the hypothesis that stereoselectivity may be a useful parameter for the functional classification of GT at the subunit level.

2. Formation of styrene-glutathione adducts by peroxidative enzymes. Several additional pieces of evidence are required to confirm our hypothesis concerning the reaction mechanism involved. Sufficient quantities of the two styrene-GSH adducts will be synthesized enzymatically, purified and chemically characterized (nmr, mass spectroscopy). This study will also be extended to determine if this reaction mechanism functions in the conjugation of other endogenous and exogenous chemicals, such as intermediates of the prostaglandin cascade and proximate carcinogenic metabolites of polycyclic aromatic hydrocarbons, with the endogenous tripeptide, GSH.

3. We hope to extend enzymatic and immunochemical analysis to freshly isolated and/or cultured vascular cells and to determine the effects of exogenous modulators on the cytochrome P-450-dependent monooxygenase system in these cells. Success in developing immunocytochemical staining for electron microscopic analysis will provide definitive evidence of the cellular and subcellular distribution of these proteins. It seems likely that an understanding of the susceptibility to toxic chemicals and the physiological and metabolic functions of various cell types will result from the determination of cellular contents, enzyme activity, and characterization of the xenobiotic-metabolizing enzymes which they contain. We are applying this approach to the endothelium of the aorta.

4. Glutathione status of lung. Our studies with PQ in perfused rabbit lung and isolated lung cells are now complete. We will investigate GSH biosynthesis in the perfused rabbit lung using radiolabeled precursors for comparison to the data obtained in freshly isolated type II and Clara cells and alveolar macrophages.

5. Trachea as a site of P-450-dependent monooxygenase activity. Immunochemical quantitation of the P-450 isozymes present in tracheal microsomes and in tracheal cells will be determined in collaboration with Drs. Domin and Philpot, LP. The metabolic data obtained with BP in our tracheal preparations will then be quantitatively compared to data obtained with the major P-450 isozymes of trachea in reconstituted monooxygenase systems.

We also hope to establish the source of the extracellular membranes in trachea which contain the major components of the P-450 monooxygenase system. These may be debris resulting from normal cell turnover or perhaps they are a secretory product. We will also determine the involvement of these membranes, if any, in pulmonary monooxygenase activity. Catalytic activity will obviously require the availability of cofactor and substrate(s).

6. The renal UDPGT will be further purified, biochemically characterized and used for the preparation of polyclonal antibodies. These antibodies will be used for the quantitation of various UDPGT in liver, lung and kidney of rats and to determine their cellular localization in extrahepatic tissues.

3. Publications (past 18 months)

Foureman, G.L., and Bend, J.R.: The hepatic glutathione transferases of the male little skate, Raja erinacea. Chem.-Biol. Interact. 49: 89-104, 1984.

Little, P.J., James, M.O., Pritchard, J.B., and Bend, J.R.: Benzo(a)-pyrene metabolism in hepatic microsomes from untreated and 3-methylchol-anthrene-treated southern flounder, Paralichthys lethostigma. J. Environ. Pathol. Toxicol. Oncol. 5: 309-320, 1984.

Yagen, B., Ben-Zvi, Z., Foureman, G., Hernandez, O., Ryan, A.J., Cox, R.H., and Bend, J.R.: The metabolism and excretion of ^{14}C -styrene oxide-glutathione adducts administered to the winter flounder, Pseudopleuronectes americanus, a marine teleost. Identification of the corresponding S-cysteine derivatives as major urinary metabolites. Drug Metab. Disp. 12: 389-395, 1984.

Bend, J.R., Foureman, G.L., Ben-Zvi, Z., and Albro, P.W.: Heterogeneity of hepatic aryl hydrocarbon hydroxylase activity in feral winter flounder: Relevance to carcinogenicity testing. Monog. Natl. Cancer Inst. 65: 359-370, 1984.

Pritchard, J.B., and Bend, J.R.: Mechanisms controlling the renal excretion of xenobiotics in fish: Effects of chemical structure. Drug Metab. Rev. 15: 655671, 1984.

Bend, J.R., and Foureman, G.L.: Variation of hepatic aryl hydrocarbon hydroxylase and 7-ethoxyresorufin O-deethylase activities in marine fish from Maine: Evidence that monooxygenase activities of only a few species are induced by environmental exposure to polycyclic aromatic hydrocarbon-type compounds. Marine Environ. Res. 14: 405-406, 1984.

Bend, J.R., and Serabjit-Singh, C.J.: Xenobiotic metabolism by extrahepatic tissues: Relationship to target organ and cell toxicity. In Mitchell, J.R. and Horning, M.G. (Eds.): Drug Metabolism and Drug Toxicity. New York, Raven Press, 1984, pp. 99-136.

Little, P., James, M.O., Pritchard, J.B., and Bend, J.R.: Temperature-dependent disposition of benzo(a)pyrene in the spiny lobster, Panulirus argus. Toxicol. Appl. Pharmacol. 77: 325-333, 1985.

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Serabjit-Singh, C.J., Bend, J.R., and Philpot, R.M.: Cytochrome P-450 monooxygenase system: Localization in smooth muscle of rabbit aorta. Mol. Pharmacol., in press.

Little, P.J., James, M.O., Foureman, G.L., Weatherby, R.P., and Bend, J.R.: 1-¹⁴C-Hexadecane disposition in the spiny lobster, Panulirus argus and the American lobster, Homarus americanus. J. Environ. Pathol. Toxicol. Oncol., in press.

Philpot, R.M., Domin, B.A., Devereux, T.R., Harris, C., Anderson, M.W., Fouts, J.R., and Bend, J.R.: Cytochrome P-450-dependent monooxygenase systems of lung: Relationships to pulmonary toxicity. In Boobis, A.R., Caldwell, J., de Matteis, F., and Elcombe, C.R. (Eds.): Microsomes and Drug Oxidations. London, Taylor & Francis, 1985, pp. 248-255.

Bend, J.R., and Serabjit-Singh, C.J.: Comparative biochemistry of the lung: Importance of cellular heterogeneity. Symposium on Problems of Drug-Related Damage to the Respiratory System. Bundesgesundheitsamt, Institut für Arzneimittel, 1985, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 80031-09 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Altered Membrane Function in Xenobiotic Toxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: J.B. Pritchard

Research Physiologist

LP NIEHS

Others: D.S. Miller

Expert Research Physiologist

LP NIEHS

COOPERATING UNITS (if any)

University of Florida, C.V. Whitney Laboratory; Duke University

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION

NIEHS/NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

3.0

PROFESSIONAL

1.3

OTHER

1.7

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The ability to transport solutes across epithelial membranes is vital for the function of many organs, e.g., secretion and reabsorption by the kidney. In turn, epithelial transport depends upon the coordinated function of individual transport systems located at opposite poles of the cells in the apical (BBM) and basolateral (BLM) membranes. Many of these membrane processes, particularly for anions, are not yet understood. Furthermore, because of their complex organization, functional importance, and exposed location, epithelial membranes are particularly susceptible to toxic effects of foreign chemicals. Our major recent emphasis has been on increasing our understanding of vectorial solute transport in polar epithelia, including the properties of specific carrier systems, the driving forces energizing transport, the regulation of transport events, and the coupling between events at opposite poles of the cells. Results for both organic (glucose, amino acids, organic acids) and inorganic (Na^+ , Cl^- , SO_4^-) solutes emphasize the complexity of the chains of events which lead to active solute transport by epithelia and thus indicate that there are multiple sites for potential disruption by xenobiotics. Moreover, they also show that similar chains are responsible for transport of widely different solutes; thus, that the same mechanism may be responsible for breakdown in transport of unrelated solutes. For example, the organic cation, L-lysine, and the inorganic anion SO_4^- , were shown to be transported via pH gradient-dependent mechanisms. Therefore, transport of both solutes could be markedly inhibited by the protonophore, pentachlorophenol, which collapses the pH gradient across the membrane.

B. PROJECT DESCRIPTION

ROLE OF ALTERED MEMBRANE FUNCTION IN XENOBIOTIC TOXICITY1. Research Project

Nature of problem: The capacity to transport solutes across epithelial membranes is vital for the function of many organs, i.e., kidney, intestine, gill. In turn, transepithelial solute transport depends upon the coordinated function of individual transport systems located at opposite poles of the epithelial cells in the apical (BBM) and basolateral (BLM) membranes. Many features of these processes, including their coupling to metabolic events, the coordination between events at opposite cellular poles, and the regulation of these processes are poorly understood, particularly for anionic solutes. Furthermore, because of their complex organization, functional importance, and exposed location, epithelial membranes are particularly susceptible to the toxic effects of foreign chemicals.

Objectives in near term:

Organic anion transport by kidney and liver mediates elimination of many xenobiotics and/or their metabolites. Transport of the inorganic anion, sulfate, controls its availability for xenobiotic detoxication through sulfate conjugation. Our first objective is to characterize the individual transport events, their coupling to metabolic energy, and the coordination of apical and basolateral events which lead to transport of these anions. We will assess the impact of these transport systems on the elimination and toxicity of potentially toxic agents and/or their metabolites. Finally, we will examine the extent to which the toxicity of foreign chemicals may be mediated through their effects on membrane transport.

Experimental design:

An important feature of our experimental design is the use of comparative models to augment the more standard mammalian techniques. We try to select a preparation which is particularly suited to the solution of a given problem. For example, the presence of an extensive renal portal circulation in fish provides ready access in vivo to the peritubular circulation of the tubules. Thus, renal clearance studies in fish are particularly suited to study the role of tubular secretion in the elimination of a given solute. Isolated flounder kidney tubules may also be easily prepared and remain viable for many hours in vitro, providing a simple yet powerful preparation for the study of intact tubular function. Furthermore, because marine teleost kidney is composed predominantly of proximal tubules (>90% by mass), they provide a more homogeneous tissue source for subcellular fractionation studies. Finally, the special needs of the marine teleost may lead to amplification of certain pathways, whereas the roles of others is diminished. In these instances, e.g., sulfate transport, such specialization may be helpful in assessing the relative contribution of different membrane events to the overall handling of a given solute.

The second important feature of our approach is the use of preparations addressing membrane function at several levels of organization from the isolated membrane to the intact animal. Thus, whereas much of our current work utilized isolated BBM or BLM vesicles because of the exquisite control they permit over the local environment of the membrane and their utility in dissecting individual membrane events. We have continued to use intact animal and isolated tubule preparations in concert with the vesicles to follow intergration of membrane events into overall epithelial function.

Recent accomplishments:

Our primary goal is to increase understanding of vectorial solute transport by polar epithelia. Major recent emphasis has been on anion transport, focusing on two systems which have direct impact on xenobiotic toxicity -- the organic anion and sulfate transport systems. Our previous work had demonstrated that the pH gradient driven transport at the BLM of the teleost renal tubule was coupled to anion exchange at the luminal membrane (BBM) to produce net tubular secretion. In the mammal, sulfate shows bidirectional transport (i.e., both secretion and reabsorption) with net reabsorption. Plasma sulfate represents the major depot of sulfate for use in sulfate conjugation reactions, and in man, sulfate availability has been shown to limit detoxication via sulfate conjugation. Therefore, the renal handling of sulfate, i.e., the balance between secretion and reabsorption, plays a critical role in determining the toxicity of many xenobiotics. Previous work, in part from this laboratory, demonstrated that reabsorption was driven by Na/SO_4 cotransport at the BBM coupled to anion exchange at the BLM. The basis for secretory sulfate flux was unknown. We have now shown the existence of an Na independent carrier in the BBM which, in conjunction with the anion exchanger at the BLM, may mediate the secretory sulfate flux. This carrier is an anion exchanger. Effective counterions include bicarbonate, chloride and to a lesser extent other monovalent anions. Transport is electroneutral, implying exchange of two monovalent anions for a single divalent sulfate anion or one monovalent anion for one sulfate and one proton. Monovalent anion gradients are equally effective in driving either sulfate influx or efflux, depending solely on the orientation of the anion gradient, i.e., the carrier is symmetrical. Divalent anions do not effectively drive either uptake or efflux of sulfate but are potent inhibitors of monovalent anion driven flux. These results indicate that monovalent and divalent anions interact with separate sites, and that both species must be present for effective transport. Also consistent with the existence of separate sulfate and monovalent anion sites is the ability of SITS, a potent inhibitor of anion exchange, to inhibit only when present on the same side of the membrane as sulfate. Finally, both inhibition and kinetic data clearly demonstrate that the BBM anion exchanger is distinct from the Na/SO_4 cotransporter also present in BBM. Thus, the secretory component of the mammalian kidney is mechanistically analogous to that of the marine teleost. Future work must

address the control of the relative magnitude of secretory and reabsorption fluxes and the influence of the renal handling of sulfate on the size of the sulfate pool in control and xenobiotic stressed animals.

The extensive renal portal system of the marine teleost provides an experimental system in which the role of tubular secretion on the handling of foreign compounds may be readily studied in vivo. Results obtained for benzo(a)pyrene (BP) and several of its phase I metabolites demonstrated the impact of both organic anion transport and sulfate conjugation. The efficacy of excretion was shown to depend upon their conversion to anions which were substrates for the organic anion system, primarily sulfate and glucuronide conjugates. Although both conjugates were transported and eliminated much more rapidly than the parent molecules, the sulfate conjugates were clearly better substrates for organic anion transport and excretion than the corresponding glucuronides. Furthermore, isolated renal tubules were capable of effective sulfate conjugation. Thus, both regulation of sulfate availability and sulfate conjugation occur at the same site, the kidney.

Our final project dealt with epithelial transport of another anion, chloride. Chloride transport by the mammalian kidney and the gill of aquatic organisms plays a primary role in osmotic and ionic regulation. Thus, its disruption by disease or xenobiotics has far reaching consequences. Unfortunately, the mechanism(s) of chloride transport are only partially understood. Mammalian kidney has a furosemide sensitive, electro-neutral Na/K/2Cl cotransport system. Teleost and crustacean gills have a second system which is not directly related to sodium. We have prepared isolated membrane vesicles from blue crab gills. These membranes transport chloride by an anion exchange mechanism which is not influenced by sodium or furosemide. It is inhibited by SITS and phloretin -- inhibitors of anion exchange in many tissues -- and is capable of moving chloride in response to bicarbonate or hydroxyl gradients. The means by which exchange is coupled to metabolic energy to produce accumulation of Cl⁻ against its concentration gradient has not yet been established. However, in view of the sensitivity of both anion exchange and anion-stimulated ATPase (An⁻-ATPase) to thiocyanate, it is possible that An⁻-ATPase might couple Cl⁻ flux to ATP hydrolysis in analogy to the Na,K-ATPase which energizes Na⁺ transport. We have begun to approach this latter possibility from two directions. First, we have shown that ATP, but not its nonhydrolysable analogs, will accelerate Cl⁻ transport by isolated vesicles. Second, we have partially purified the An⁻-ATPase and prepared monoclonal antibodies against it. The antibodies will be used to study its localization within the cell (a matter of significant controversy at present), its induction in response to increased osmoregulatory stress, and its involvement in chloride transport.

2. Plans for Future

Development of isolated membrane vesicle techniques has facilitated characterization of the individual transport events and disclosed the complexity of the events which couple transport of a given solute to the metabolic energy and lead to net solute movement across the epithelium. We propose to use a combination of vesicle and intact tubule techniques to address the renal handling of both organic and inorganic solutes. Our focus will be twofold: a) to characterize the membrane basis for the handling of anions which play a role in xenobiotic toxicity (sulfate, organic anions), and b) to determine the extent to which toxicity may be related to alteration in membrane function in epithelial systems.

Many of the characteristics of the individual carriers responsible for anion transport (organic anions, sulfate, and chloride) have been largely determined. However, other important features including the means of coupling to metabolic energy, the coordination of events at opposite cellular poles, and the modulation of transport in response to physiological state or xenobiotic stress remain to be clarified. Initially, membrane vesicle and intact cell preparations will be used to evaluate possible means of energy coupling, including direct coupling to ATP hydrolysis or indirect coupling via ion (particularly Na^+) or potential gradients. In addition, purification and immunochemistry will be utilized to evaluate the possible role of An^- -ATPase in transport. Finally, we will attempt to modify techniques developed to measure intracellular solute activities in individual oocytes for application to the smaller cells of epithelia. If successful, these techniques will allow us to examine directly the interrelationships between membrane events and cellular ultrastructure and compartmentalization.

Current emphasis will remain on sulfate transport in teleost and mammal since we have the most complete picture of its handling in both species. However, since many of its features are shared by the organic anion system, we will attempt to extend our findings to this system as well. Here our aim will be not only to understand the mechanism of organic anion transport and its interactions with sulfate, but also to understand those features of the organic anion system which are critical in determining its efficacy in the elimination of foreign compounds.

3. Publications (past 18 months)

Little, P.J., James, M.O., Pritchard, J.B., and Bend, J.R.: Benzo(a)pyrene metabolism in hepatic microsomes from untreated and 3-methylcholanthrene-treated southern flounder, *Paralichthys lethostigma*. J. Environ. Pathol., Toxicol., and Oncol. 5: 309-320, 1984.

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Pritchard, J.B., and Bend, J.R.: Mechanisms controlling the renal excretion of xenobiotics in fish: Effects of chemical structure. Drug Metab. Rev. 15: 655-671, 1984.

Little, P.J., James, M.O., Pritchard, J.B., and Bend, J.R.: Temperature-dependent disposition of ^{14}C -benzo(a)pyrene in the spiny lobster, Panulirus argus. Toxicol. Appl. Pharmacol. 77: 325-333, 1985.

Lee, S.-H., and Pritchard, J.B.: Bicarbonate/chloride exchange in gill plasma membranes of the blue crab. Am. J. Physiol., in press.

Pritchard, J.B., and Bend, J.R.: Role of secretory transport in the renal excretion of benzo(a)pyrene and its metabolites. Proc. Third International Symposium on "Responses of Marine Organisms to Pollution," in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 80038-02 LP									
PERIOD COVERED October 1, 1984 to September 30, 1985											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Suicide Inhibitors of Cytochrome P-450: Isozyme and Tissue/Cell Selectivity											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: John R. Bend</td> <td style="width: 30%;">Chief</td> <td style="width: 30%;">LP NIEHS</td> </tr> <tr> <td>Others: J. Mathews</td> <td>Staff Fellow</td> <td>LP NIEHS</td> </tr> <tr> <td>G. Parker</td> <td>Chemist</td> <td>LP NIEHS</td> </tr> </table>			PI: John R. Bend	Chief	LP NIEHS	Others: J. Mathews	Staff Fellow	LP NIEHS	G. Parker	Chemist	LP NIEHS
PI: John R. Bend	Chief	LP NIEHS									
Others: J. Mathews	Staff Fellow	LP NIEHS									
G. Parker	Chemist	LP NIEHS									
COOPERATING UNITS (if any)											
LAB/BRANCH Laboratory of Pharmacology											
SECTION Molecular and Comparative Pharmacology											
INSTITUTE AND LOCATION NIEHS/NIH, Research Triangle Park, North Carolina 27709											
TOTAL MAN-YEARS 2.2	PROFESSIONAL 1.2	OTHER 1.0									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews											
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided) <p> <u>Microsomal monooxygenase systems</u> contain multiple <u>isozymes of cytochrome P-450</u> which contribute differentially to the oxidative metabolism of endogenous and exogenous substrates; isozyme differences in K_m, V_{max}, regioselectivity and stereoselectivity are common. Hence, modulation of the relative amounts of various P-450 isozymes can have pronounced effects on chemical metabolism and toxicity. For this reason we are studying isozyme selectivity and <u>tissue/cell selectivity of suicide inhibitors of cytochrome P-450</u>. The suicide inhibition by <u>1-aminobenzotriazole</u> (ABT) and some of its novel N-alkylated derivatives, which we synthesized and characterized, is being studied in rabbit lung and liver. Although ABT is a potent suicide inhibitor, it shows little P-450 isozyme selectivity. N-benzyl-ABT, on the other hand, is both potent and highly selective (but still not specific; it destroys isozymes 2 and 6 associated enzyme activity but does not significantly affect isozyme 5 catalyzed activity). In a related project, the chemical nature of the <u>alkyl-benzene metabolites</u>, which selectively destroy pulmonary (versus hepatic) cytochrome P-450, and the biochemical nature (e.g., isozyme specificity, involvement of other enzymes and cofactors) of the pathways involved are being elucidated. Results to date demonstrate at least two distinct pathways for metabolic activation, one relying only on the presence of NADPH for suicide destruction, the second relying both on the presence of NADPH and alcohol dehydrogenase. </p>											

B. PROJECT DESCRIPTION

Suicide Inhibitors of Cytochrome P-450: Isozyme and Tissue/Cell Selectivity1. Research Project

Nature of problem: Microsomal monooxygenase systems generally contain multiple forms of cytochrome P-450, each of which may contribute to the overall oxidative metabolism of a substrate in a different manner; isozyme differences in regioselectivity, stereoselectivity, K_m and V_{max} are common. Consequently, modulation of the relative amounts of P-450 isozymes can have pronounced effects on chemical metabolism, and on toxicity. The administration of different enzyme inducers to animals results in the selective induction of certain P-450 isozymes and the repression of others. Suicide inhibition of cytochrome P-450 offers another potential method for affecting this monooxygenase system in an isozyme and tissue selective manner. Such inhibition, if isozyme specific, will allow the assessment of the catalytic functions of the remaining P-450 isozymes for both endogenous and exogenous substrates. This experimental approach should be equally valid in systems with intact cellular structure and in microsomal preparations.

Objectives in near term (hypothesis tested):

1. To determine the isozyme and tissue/cell selectivity of the suicide inhibition of cytochrome P-450 mediated by the metabolism of 1-aminobenzotriazole and some of its N-substituted derivatives, and to utilize such isozyme selective inhibition to study xenobiotic metabolism in systems with intact cellular structure. (Specific, non-toxic suicide inhibitors offer the potential for protection of humans against specific chemicals to which they are exposed, or by co-administration, for enhanced toxicity of pesticides. In any event this experimental approach will be useful for delineating physiological functions of cytochrome P-450 at the isozyme level, our primary interest).

2. To delineate the chemical nature of the reactive metabolite(s) of alkylbenzenes, such as p-xylene, which selectively destroy pulmonary versus hepatic cytochrome P-450, and to determine which P-450 isozymes are responsible for this suicide catalysis (alkylated benzenes are common chemicals of commerce and chemical/biochemical aspects of their metabolic activation/detoxication pathways require thorough investigation).

Experimental approach and scientific justification:

An integrated experimental approach is used and studies are conducted with purified P-450 isozymes, subcellular fractions of various tissues, freshly isolated or cultured cells, isolated perfused organs and with intact animals. Required novel inhibitors and metabolites are synthesized in the

laboratory, and they are characterized by NMR and mass spectroscopy. Metabolite isolation, identification and quantitation are most frequently accomplished by high performance liquid chromatography and scintillation counting or by gas chromatography. Cytochrome P-450 loss is determined by spectral measurement and inhibition of catalytic activity is determined by the use of isozyme selective or specific substrates (e.g., benzphetamine N-demethylation for isozyme 2, N-hydroxylation of 2-aminofluorene for isozyme 5 and 7-ethoxyresorufin deethylation for isozyme 6 in rabbit lung). This experimental approach allows us to investigate the chemical and biochemical basis of P-450 isozyme selective suicide inhibition.

Recent accomplishments/significance:

1. ABT and its analogues. To better understand the mechanism of destruction of cytochrome P-450 by 1-aminobenzotriazole (ABT) in the perfused rabbit lung, several experiments were performed with ^{14}C -ABT. We demonstrated that the pulmonary uptake of ABT from perfusion medium is low as is its metabolism in the isolated organ (more than 95% of the parent compound was recovered after a 60 min perfusion). After perfusion, approximately 0.3 nmol ABT equivalent/mg microsomal protein of covalently bound radioactivity was found which approximates the loss of cytochrome P-450 (0.3 nmol/mg microsomal protein). No ABT-mediated effect was noted on the flavin-containing monooxygenase system (FCM) even after perfusion with 1 mM ABT for 60 min, conditions which destroy up to 90% of the total P-450 present. Collectively, results of our experiments demonstrate that even in the absence of facilitative transport into the lung, ABT is an effective suicide inhibitor of P-450, that ABT can effectively inhibit the P-450-dependent monooxygenase system without affecting the FCM, and that ABT metabolism by non-P-450 pathways in perfused lung is minimal.

α -Methylbenzyl-1-aminobenzotriazole (α -MB), an N-substituted analogue of ABT that is sterically more hindered about the nitrogen atom that is a target for oxidation than N-benzyl-ABT (BBT), was synthesized and characterized by exact mass spectrometry and NMR. Dose-response data were obtained for the suicidal destruction of cytochrome P-450 and inactivation of P-450 isozyme 2-dependent benzphetamine N-demethylation and isozyme 6-dependent 7-ethoxyresorufin O-deethylation in pulmonary microsomes prepared from β -naphthoflavone-induced rabbits with ABT, BBT and α -MB. We found that ABT inactivated all three major P-450 isozymes (forms 2, 5 and 6) present in these microsomes. BBT was more potent than ABT (up to 100-fold), and was completely and equally effective at destroying form 2 and form 6 catalyzed activity; BBT did not inactivate isozyme 5 even at a concentration of 1 mM. α -MB is the most potent and selective suicide inhibitor of this series of chemicals. At a concentration of 1 μM , it destroys approximately 80% of isozyme 2, about 20% isozyme 6 and does not affect isozyme 5. At higher concentrations (up to 1 mM), more than 90% of isozymes 2 and 6 are inactivated but isozyme 5 is spared. Preliminary results

with BBT administered via the lateral ear vein of rabbits at a dose of 10 μ mol/kg demonstrated that up to 90% of P-450 form 2 was destroyed in lung. Our results indicate that BBT and especially α -MB have potential as isozyme and tissue-selective suicide inhibitors of cytochrome P-450.

2. Alkyl benzenes. Methylated benzenes with 1 to 6 methyl groups were tested for their ability to destroy (spectrally) rabbit pulmonary cytochrome P-450 in the presence of NADPH. Of the many congeners tested, only 1,2,3-trimethylbenzene was a potent suicide inhibitor (40-80% destruction).

2. Plans for Future

1. ABT and its analogues. The alkylated porphyrin resulting from heme modification during destruction of cytochrome P-450 by BBT and will be isolated and chemically characterized to delineate the mechanism of suicide inhibition. One additional compound, the very sterically hindered N-t-butyl ABT will be synthesized and tested for its relative ability to inactivate rabbit P-450 forms 2 and 6. From data obtained with BBT and α -MB, we predict that the t-butyl derivative will be more selective than BBT or α -MB for the destruction of form 2 vs. 6. We also intend to resolve (\pm)- α -MB to determine the relative activities of the two enantiomers as suicide inhibitors of P-450 isozymes 2 and 6. ABT, BBT and α -MB will be tested for inhibitory characteristics with FCM purified from pig liver. Finally, the ABT analogue which shows the highest P-450 isozyme selectivity will be tested for its ability to inhibit the functions of individual isozymes in vivo or in perfused organ preparations.

2. Alkyl benzenes. The major 1,2,3-trimethylbenzene metabolites formed in the pulmonary monooxygenase system will be chemically characterized to determine the fate of the ring and methyl groups as either free metabolites or covalent adducts to the heme prosthetic group of P-450 during the inactivation of pulmonary P-450.

C. Publications (past 18 months)

Mathews, J.M., and Bend, J.R.: Analogs of 1-aminobenzotriazole (ABT) as isozyme selective inhibitors of rabbit pulmonary cytochrome P-450 (P-450). Fed. Proc. 44: 1466, 1985.

Mathews, J.M., Dostal, L.A., and Bend, J.R.: Inactivation of rabbit pulmonary cytochrome P-450 in microsomes and isolated perfused lungs by the suicide substrate 1-aminobenzotriazole. J. Pharmacol. Expt. Ther., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 80039-02 LP
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Xenobiotic Transformation in Isolated Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	James R. Fouts	Research Pharmacologist LP NIEHS
Others:	Theodora Devereux	Research Biologist LP NIEHS
	Thomas Massey	Visiting Fellow LP NIEHS
	Janet Diliberto	Biological Lab. Technician LP NIEHS
	Blair Hoyle	Biological Lab. Technician LP NIEHS
	Thomas Eling	Research Chemist LMB NIEHS
	Richard Philpot	Research Chemist LP NIEHS
COOPERATING UNITS (if any) Biometry and Risk Assessment Program (BRAP); Histology, NIEHS; Department of Pulmonary Medicine, University of North Carolina School of Medicine, Chapel Hill, N.C. (human tissues)		
LAB/BRANCH Laboratory of Pharmacology		
SECTION Cell Pharmacology		
INSTITUTE AND LOCATION NIEHS/NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.6	2.0	2.6
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Major cell types of the lung are being isolated and studied for their metabolisms of xenobiotics and selected lipids (in comparison with liver cells). Special studies are being made with Clara, type II and ciliated cells of the lung. Studies of the effects of the techniques used in isolating the enriched populations of cells from tissues are being made with various antibodies to selected cytochrome P-450 isozymes and Western blotting/microdots for quantifying these isozymes and related peptides in cells at various stages in the isolation procedures. A microspectrophotofluorometer is being used to quantify xenobiotic metabolism in single cells and to study variations in this metabolism among cells of an "homogenous" population. Variations in enzyme activity in periportal and centrolobular liver cells in the perinatal period are compared with these activities in the adult liver. Lung cells from dogs and humans are being prepared and analyzed for P-450 isozymes and xenobiotic metabolism in comparison with cells from the rabbit.		

B. PROJECT DESCRIPTION

XENOBIOTIC BIOTRANSFORMATION IN ISOLATED CELLS1. Research Project

Nature of problem: Damage to tissues by chemicals is often localized in one part of the tissue or varies in intensity from one area of the tissue to another. With many chemicals, cellular damage is dependent on the metabolism of the chemical by enzymes in the target cells. Localized or area-specific damage implies that different cells or cells in different parts of the organ may have different chemical metabolizing systems. Our research is directed to analyzing and quantifying chemical metabolisms in different cell types and among cells of one type. We have been studying these chemical metabolizing systems in 3 organs: lung, skin, and liver, and have shown that our basic hypothesis is true in each organ -- different types of cells do have different amounts of chemical metabolizing enzymes. The present work will continue these studies and also correlate the chemical metabolizing activities with specific damages to cells by selected chemicals (whose effects depend on metabolism). Studies of effectors of the type and amount of chemical metabolism in specific cell types and organs are also being made (e.g., animal pretreatments with chemicals/drugs to induce or inhibit metabolism; variations in metabolism in the perinatal period). Our recent studies have mostly concerned metabolizing systems in isolated cells of the lung. Isolated cells may be able to more exactly answer questions about individual cell metabolic capacities and regulations than studies on the whole tissue (as with histochemical methods), and cell-cell interactions may be studied with greater control of variables. Isolated cells do quickly change in enzyme composition (especially with regard to chemical metabolizing systems of certain types), and it is necessary to study such changes and how these may obscure the comparison of results with the in vivo situation. Our studies therefore focus on these problems too: 1) isolating enriched populations of specific cell types and 2) studies of changes in enzymes in isolated cells and comparison with cells in the tissue.

There have been three major sub-projects active in the last year: 1) characterization of chemical metabolizing systems in Clara and type II cells of the rabbit lung; 2) development of methods for isolating ciliated cells and Clara cells from upper airways of rabbits and a study of their chemical metabolisms; 3) developing the microspectrophotometer for study of metabolism of benzo(a)pyrene by single isolated liver cells. A fourth project is just beginning -- developing methods for isolating type II (and, if possible, Clara cells) from human and dog lung -- as provided by collaborators in the pulmonary function division at N.C. Memorial Hospital. This project, if successful, will then continue by studying xenobiotic metabolisms and P-450 isozyme contents in the isolated cells, and comparison with rabbit cells. With enough samples, it may be possible to correlate disease

and/or smoking history with cell enzyme activity in the human materials. A collaboration with Dr. Klinger from the Institute for Pharmacology and Toxicology in Jena (East Germany) continues. This project began during Dr. Klinger's study here in 1983. The thesis is that cells of the periportal versus centrilobular areas of liver will develop xenobiotic metabolizing systems at different rates in the perinatal period. Successful development of the microspectrophotometer as a tool to study xenobiotic metabolism by single liver cells will help in this project.

Objectives in near term:

a) The lung cells:

1. Characterize the xenobiotic and endogenous substrate metabolizing enzymes of the Clara and alveolar type II cells:

- aa) Determine and quantify cytochrome P-450 isozymes in these cells in the rabbit lung.
- bb) Study steroid and lipid metabolisms (progesterone, glucocorticoids, prostaglandins and arachidonic acid) in these cells by cytochrome P-450s and other enzymes.
- cc) Study effectors of these systems -- inducers and inhibitors -- especially with regard to the balance of toxication/detoxication systems, amounts of specific P-450 isozymes, specific metabolites (e.g., of benzo(a)pyrene (BP)) produced in induced type II and Clara cells.

2. Isolate, enrich and characterize fractions of other lung cell types:

- aa) Isolate the ciliated cells from upper airways and enrich them in fractions for enzyme characterizations -- especially to allow for distinction between ciliated versus Clara and type II cells.
- bb) Quantify cytochrome P-450 isozymes (and xenobiotic metabolisms dependent on these) in ciliated cells of the rabbit trachea/upper bronchi. Compare Clara cells from two parts of the rabbit airway (upper versus lower) -- metabolically and types/quantity of P-450 isozyme(s).

3. Isolate, enrich and characterize cells from dog and human lung samples:

- aa) Isolate type II cells and, if possible Clara cells, from lung samples from dogs and humans. Characterize these by histology and electron microscopy, xenobiotic metabolisms, and prostaglandin and arachidonic acid metabolisms.
- bb) Attempt to identify and quantify the P-450 isozymes in these cells and compare enzyme activity, xenobiotic metabolite patterns in type II cells (or Clara cells) from dog and human with the rabbit as a function of P-450 isozyme contents.

cc) With enough samples of human lung, attempt to correlate metabolisms, activities, metabolites with disease or smoking history.

b) The skin cells (if this project resumes:

1. Characterize the xenobiotic and endogenous substrate metabolizing enzymes of the different cell layers:

- aa) Determine the type and amount of prostaglandin metabolisms in the cell layers.
- bb) Attempt to quantify P-450 isozymes in different cell layers.
- cc) Attempt to enrich particular cell types of the keratinocytes.

2. Study the distribution of administered chemicals (e.g., PCBs) in different skin cells.

c) The liver cells:

1. Continue the projects describing the perinatal development patterns of xenobiotic metabolism in periportal versus centrolobular areas.

2. Use isolated hepatocytes to study xenobiotic metabolism in single cells with the microspectrophotofluorometer:

- aa) To develop methods for quantifying enzyme activity in single cells.
- bb) (With these methods) to study the variability of enzyme activity in single cells and from various parts of the liver lobule -- before and after exposure of animals to inducers/inhibitors of xenobiotic metabolism.

d) Hypotheses to be tested:

1. Individual cells of the same type may vary in enzyme activity depending on location in the tissue (e.g., periportal versus centrolobular hepatocytes; Clara cells in upper versus lower airways); and this variation may even be enhanced by animal treatments (e.g., after inducers/inhibitors) or at different ages (newborn versus adult). These variations can translate into susceptibility to damage by chemicals metabolized to active fragments (e.g., CCl_4 in liver; ipomeanol in lung).

2. Lung cells rich in (lipid) inclusion bodies will metabolize steroids and lipids like prostaglandins, arachidonic acid at much different rates from cells without or with few such bodies (e.g., type II cells versus Clara cells versus ciliated cells). Cells rich in endoplasmic reticulum, especially smooth-surfaced, will have higher P-450-dependent metabolisms (chemicals and lipids) than cells poor in ER (e.g., Clara versus type II and ciliated cells; Clara cells of upper versus lower airways).

3. P-450 isozymes will differ in amount and type from one lung cell type to another. Different cell types may have different stability of the P-450 forms (type II versus Clara cell form 2 of P-450 may be different) or P-450 turnover may be different in different cells.

4. Lung cells from the human will behave in ways that are similar to those of experimental animals already tested -- allowing methods to be adapted for isolation of type II cells and testing these cells for xenobiotic metabolisms and responses to inducers of P-450 isozymes (such as smoking). Lung cells from diseased lungs will metabolize xenobiotics and lipids (arachidonic acid/prostaglandins) at rates different from those of healthy lungs.

Experimental approach and scientific justification:

a) The lung cells: Methods for isolating enriched fractions of Clara and type II cells from both rat and rabbit lungs have been published and these will be used in further studies. The enzyme pathways to be studied include 7-ethoxycoumarin (7-EC) deethylation, 7-ethoxyresorufin (7-ERF) deethylation, coumarin hydroxylation, benzphetamine demethylation as well as those specific for selected isozymes of P-450 (e.g., 2-acetylaminofluorene hydroxylation, BP hydroxylations, progesterone and testosterone hydroxylations or key enzymes in arachidonic acid or prostaglandin metabolism). Metabolism of most steroids has been followed by TLC methods using radioactive substrates and comparisons with known metabolite standards for quantification. This has worked well for estrogen and progesterone metabolism studies, and will be used when we study the glucocorticoids. If large amounts of unknown metabolites are produced (we seem to get this with progesterone and the Clara cell), we will use HPLC for better separation and preparation of material for identification. Prostaglandin and arachidonic acid metabolisms will be followed first in collaboration with Dr. Eling using HPLC (this is being done with the lung cells, and can be done with the skin cells).

Antibodies to specific P-450 isozymes and to cytochrome P-450 reductase have been used for inhibition and quantification studies (e.g., the microdot/blotting techniques for measuring forms 2, 5 and 6 of pulmonary P-450 in isolated cells and fractions of cells). These techniques applied to cells at different stages in the preparation/isolation procedures can help us to detect changes in amount and nature of P-450 in cells (e.g. proteolysis and possible causes for this during isolation of Clara cells; the protective effects of added anti-proteases on P-450 forms in different cell types). These techniques can also be used to determine differences in cell content of P-450 isozymes after animal treatments (e.g., inducers), or between one cell type and another (Clara cells at different parts of the trachea/lung; Clara versus ciliated or type II cells).

These approaches combine measurement of enzyme activity and isozyme content with histology and electron microscopy of the fractions at the time of measurement. The methods used and their application are chosen to uncover differences in xenobiotic and endogenous substrate metabolisms in major lung cell types and will be the basis of understanding the role of these metabolisms in chemical damage to the lung as well as possible biochemical and physiological functional differences among lung cells and the relations of these to cell location, cell structure, and interactions.

The cells chosen for study (type II, Clara, ciliated and macrophages) are all major lung cell types and are postulated to play roles in chemical metabolisms in the lung. The study of lipid metabolisms and the choice of steroids to study is based on the role of these cells in lipid storage/release in the lung (type II cells produce, store and secrete surfactant) and on the response of these cells to various lipids/steroids (glucocorticoids cause proliferation of surfactant/type II cells; prostaglandins affect ciliated cell activity).

Besides rabbit lung cells, human and dog lung cells will be studied for xenobiotic and selected lipid substrates' metabolisms. The type II cells can be isolated from both dog and human lung samples by adapting techniques used for the rabbit lung. Clara cells may not be isolatable -- they are in small quantities in both species, and especially after smoking. Preliminary studies are in progress now using lung pieces obtained from lung resections by the Department of Pulmonary Medicine, University of North Carolina Medical School, Chapel Hill, N.C. There are marked differences in type II and Clara cell morphologies among species and this usually translates into differences in preparation/isolation and metabolism. We hope to test these possibilities and see how closely human and dog tissue resembles those of other animals we have been using so far. Prostaglandin metabolism by human and dog cells will be studied in collaboration with Dr. Eling while quantification of P-450 isozymes and studies of possible changes in xenobiotic metabolism with cell isolation and fractionation will be studied in collaboration with Dr. Philpot.

b) The skin cells (if this project resumes): Methods for separating skin cells into fractions corresponding to layers in the skin have been developed for the hairless mouse. The pathways studied will include selected xenobiotics and steroids/lipids to pinpoint enzyme differences (e.g., P-450 isozymes). If antibodies to rabbit lung P-450s work on mouse skin (P-450s), we may be able to quantify forms of P-450 -- otherwise we will use substrate specificity until antibodies are available (various substrates which are specific for P-450 forms, as described for the lung cells, above).

The studies to be completed here will further characterize the metabolic capacities of different layers of the skin and point to those layers and possible specific cell types with high versus low chemical metabolisms. The prostaglandin/lipid/steroid metabolisms will be different in cells

which store lipids (e.g., sebaceous cells) versus those not having such functions (e.g., basal cells). Alterations in lipid storage/metabolism are produced by a number of environmental toxins active on the skin -- e.g., PCBs, dioxins. Specific effects of such toxins on particular cells and on particular types of lipid metabolism can be studied using these isolated cells to more precisely pinpoint sites of action and metabolisms affected.

c) The liver cells: Hepatocytes are isolated from rabbit liver using conventional techniques (collagenase perfusion). The metabolic capacities of individual hepatocytes are studied with the microspectrophotofluorometer using as substrate BP. Cells are incubated with BP to allow diffusion into the cell of this substrate. Cells are then washed to remove excess substrate and metabolism is followed by the decline in fluorescence. This is an adaptation of methods used by Lahmy *et al.* (*Toxicology* 29:345, 1984) with fibroblasts. This method can be used to study variations in enzyme activity among the "same" type of cells and the effects of animal treatment with inducers on both total metabolism and inter-cell variations in metabolism. We also hope to use these methods to follow the development of BP metabolisms in the perinatal period in the different regions of the lobule -- extending the work begun by Dr. Klinger showing that metabolism was different in pericentral versus periportal cells in the adult, but not in the newborn animal. We hope to use the microspectrophotofluorometer to see if xenobiotic metabolism develops in one lobule area before another and in all cells of an area at once. We also hope to document the inter-cell variability in BP in "normal" versus "induced" livers in adult versus young animals, which has been postulated for years. Such studies will help understand why not all cells of a particular type or location are equally affected by a given dose of toxicant whose effects depend on metabolism to active metabolites. Methods found useful with individual hepatocytes will be tested on individual pulmonary cells starting with Clara cells from rabbits (highest activity of all pulmonary cells so far isolated).

Recent accomplishments:

a) Lung cells:

1) Initial studies of xenobiotic metabolism of Clara and type II cells have been completed. A paper with some of these results has just been published (Devereux, Diliberto and Fouts, 1985).

2) Several classes of lipids are metabolized by lung cells. Progesterone is metabolized in both type II and Clara cells (greater in Clara). A large amount of non-polar metabolite in Clara cells may be 20-hydroxy-progesterone. Prostaglandin and arachidonic acid metabolism is being studied along with Dr. Eling's group. PGF₂- α and arachidonic acid are metabolized in both Clara and type II cells -- metabolites are being characterized by HPLC.

3) We are working with Drs. Domin and Philpot on a project to quantify and identify the P-450s (isozymes) in type II and Clara cells (Western blot/microdot methods). We are studying P-450 and reductase fragments (peptides from proteolysis?) in cell fractions as a function of stage of preparation, cell type and animal treatment -- in both liver and lung. We have detected P-450 forms 2, 5 and 6 in Clara and type II cells and shown changes after animal treatment with TCDD (to induce form 6). Peptides of P-450 form 2 and reductase seem to be present in fractions of lung cells (both type II and Clara cells) at all stages after the initial digestion to free cells from the lung. These peptides are not eliminated by any methods used (adding anti-proteases; P-450 ligands like nicotinamide, metyrapone; treating the animal with lysosomal stabilizers like steroids; or changing amount or type of protease, time of contact with lung, etc.) though the amounts are especially dependent on how long it takes to prepare the cells. Cytochrome c reductase seems to be more sensitive to proteolysis effects than P-450s, and measured activity of isolated cells' microsomes can be increased by adding extra reductase. With the best methods now used to isolate type II and Clara cells, we are able to get specific activities of several MFOs which are several fold higher than ever before and in excess of the specific activity of homogenates of fresh lung). With the best techniques, it is still possible to detect peptides of reductase in all cells in both liver and lung.

Recent findings in Dr. Bend's laboratory suggest that not all "peptides" of P-450 in these cells may come by degradation of intracellular P-450 (due to proteases somehow getting into cells during the isolation and fractionation procedures). Lung mucous contains immunoreactive P-450 and this could be "outside" all the cells we isolate. Proteases added to free the lung cells from the matrix will digest this extracellular P-450 and give peptides adsorbed to fragments which will sediment in the "microsomal" fraction prepared from cells for analysis. Damaged cells will also be accessible to protease action; and again P-450 peptides will result and be detected. Experiments are in progress to assess the contribution of this extracellular P-450 to the "proteolysis" which seems to be present in the best cell preparations so far. Extracellular P-450 and P-450 peptides are likely to adsorb to membranes both of intact cells and damaged cells and methods of removing this are being studied so that "pure" cells can then be lysed/sonicated to determine if peptides exist within intact cells as well.

4) In collaboration with Drs. Yankaskas and Van Scott (University of North Carolina School of Medicine, Department of Pulmonary Medicine), human and dog lung cells are being isolated for study of xenobiotic metabolisms and comparisons with other species. Human type II cells have been isolated and enriched to at least 80% purity (major contaminants are macrophages). Human type II cells and macrophages are now being studied for P-450 isozymes and associated enzymes. Dog type II and Clara cells have been isolated in at least 50% purity. Electron micrographs of cells from both

species have been made. The dog Clara cell is quite different from the rabbit -- little endoplasmic reticulum (ER) and almost no NBT staining (rabbit has much of both). Dog and human cells will be used for studying both arachidonic acid and prostaglandin metabolisms. Dog cells will not be extensively studied for P-450 isozymes or activities since dog lung has almost no detectable P-450.

5) BP metabolism and metabolite quantification in type II and Clara cells from control versus TCDD-treated rabbits has been studied using HPLC. The metabolite profiles of Clara and type II cells were similar, though Clara cells were more active in total metabolism (1.5-fold in control animals). TCDD treatment caused 2-fold increases in overall BP metabolism in both cell types. The largest increases were seen in P-450 form 6 (isozyme and associated metabolites) after TCDD (e.g., BP-9,10- and 7,8-diols).

6) A method has been developed for the preparation of enriched populations of ciliated cells from rabbit tracheas -- protease digestion and elutriation of cells gave 2 cell fractions (small cells = basal cells; versus larger cells), and further fractionation of the large cell mixture gave ciliated cells at 85% purity or better. Ciliated cells had both 7-EC deethylase and coumarin hydroxylase activity and resembled Clara cells much more than type II cells in all respects. Trace contamination by Clara cells could not account for the activity of the ciliated cell fractions.

b) Liver cells:

1) The collaborative project with Dr. Klinger continues. A paper describing the initial studies on xenobiotic enzyme distributions intralobularly during the perinatal period has been submitted to Toxicology and Applied Pharmacology and was presented at a European Symposium on perinatal maturation in Leipzig. Two other papers on this work are being prepared for submission to the German journal Experimental Pathology -- detailing studies with elutriation of cells as a method for separating periportal and centrilobular hepatocytes (works for adult, but not fetal/newborn tissues).

2) Western blotting and microdot studies with Drs. Domin and Philpot as collaborators have shown that fresh hepatocytes, prepared by the best methods now available, still seem to show significant proteolysis of both cytochrome c reductase and some forms of P-450. Liver microsomes prepared as quickly as possible from undigested liver homogenates (not cells) do not show such peptides.

3) The microspectrophotofluorometer can be used to measure the metabolism (overall) of BP by individual hepatocytes. The method used is an adaptation of one previously published (see "Experimental approach/scientific justification"). It is possible to calculate fluorescence rate constants for BP disappearance (metabolism) in individual cells and these were shown to differ from one cell to another among a population of the "same" healthy

cells (variations of the order of 2-fold) and to be affected by additions of inhibitors of P-450-catalyzed BP metabolism (ellipticine) or animal treatment with β -naphthoflavone, an AHH inducer. Cells from induced animals had much higher AHH activities. Cells with obvious damage (blebs, swelling, fuzzy membranes) had very low enzyme activities. In this project, several possible substrates of P-450 enzymes were used -- e.g., 7-EC and 7-ERF. Some of these have been used in other laboratories as measures of enzyme activity in single cells using equipment similar to ours. Our studies have shown that most of the work reported in the literature is flawed by the assumptions made -- that fluorescence measured in the cell reflects enzyme activity -- primarily that the fluorescent product will accumulate in the cell. This does not occur in most cases -- both conjugation and diffusion from the cell occur and affect the results markedly. So far we have not found any substrate whose fluorescent product can be measured that is suitable in such studies. Thus, substrate disappearance appears to be the best approach to single cell xenobiotic metabolism measurements. We have presented this work at national meetings (e.g., FASEB, April, 1985).

The significance of all these studies with both lung and liver cells is the confirmation of the hypotheses that different cell types will have different enzyme compositions and that these can be studied in freshly isolated cells. The differences in enzyme activity among the lung cell types correlates well with different susceptibilities of these cells to damage by chemicals whose toxicity depends on the activity of the enzymes we measure (e.g., CCl_4 effects on lung and liver cells due to metabolites of CCl_4 ; ipomeanol effects on Clara cells versus type II cells, etc.). Variations in enzyme activity among cells of the same type (which seems to occur with hepatocytes) may be a clue to why not all cells of a given region of the liver are damaged by low doses of intoxicants. The study of how and when cells of the perinatal liver develop the xenobiotic metabolisms we measure can be used in understanding damage and changes with age in damage to such livers by chemicals. The study of endogenous substrates of enzymes in Clara, type II and ciliated lung cells (e.g., steroids or prostaglandins, arachidonic acid) can give important clues as to functions of these cells and their role in regulating lung responses and blood flow. Comparisons of human, dog, and rat/rabbit lung cells will aid in understanding how to extrapolate results in animals to humans -- especially with regard to chemicals whose toxic lung effects are related to metabolites, and/or may be mediated/affected by prostaglandins, arachidonic acid, or steroids.

2. Plans for Future

Lung cells:

a) Continue studies on lipid metabolisms by isolated lung cells -- Clara and type II cells especially. This work is well along in the rabbit, and will be extended to the human and dog cells as these latter are prepared.

The progesterone metabolism studies will be done primarily in the rabbit, while the study of prostaglandin and arachidonic acid metabolisms will be done in all species. Type II cells will be used in all studies, but Clara cells may not be available in quantity and purity to do more than a few experiments in the case of human lung tissues.

b) Studies on changes in the P-450 isozymes in isolated lung (and other tissue) cells will continue in collaboration with Drs. Domin and Philpot. First, we will be completing the initial study of changes in P-450 isozymes (especially form 6) after TCDD treatment in rabbits and correlating this with BP metabolite changes -- in both type II and Clara cells. Then we will further study the problem of P-450 "peptides" in freshly isolated fractions of type II and Clara cells. We will be assessing the contribution of extracellular P-450s (from lung mucous and damaged cells) to the overall amounts and types of peptides seen in analyses of both lung and liver cells using the Western blot and microdot methods. The goal here is to define whether the presence of peptides indicates "degraded" P-450 systems and cells or merely "contamination" of healthy cells. Similar studies will be run with dog and human cells (and skin cells when that project restarts).

c) Continue studies begun with Drs. Mathews and Bend on the use of suicide substrates of P-450 in describing the P-450 isozyme content/composition of different cell types and correlating this with susceptibility of these cells to damage by various chemicals. The possible use of suicide substrates bearing a fluorescent residue to quantify enzymes in single cells will be studied further.

d) Continue to develop the microspectrophotofluorometer for assay of enzymes in single cells. Apply the method to the problem of single lung cells' BP metabolism (starting with Clara cells).

e) Continue study of isolation and enrichment and characterization of other lung cell types. Ciliated cells have been isolated in good yield and purity from the rabbit, and enzyme studies, characterization of P-450 isozymes and changes in these and reductase during isolation are being continued. Studies of endogenous substrates (prostaglandins, arachidonic acid, steroids) and comparisons of ciliated cells from different species will be started soon. Effects of TCDD on enzymes in ciliated cells from rabbits will be studied using both substrates and P-450 antibodies along the lines used with Clara and type II cells. Clara cells from upper airways will be compared with Clara cells from lung parenchyma in the rabbit to see how cell location affects enzyme composition, morphology, etc. The small cell fractions prepared from upper versus lower airways (basal cells?) in the rabbit will also be compared with regard to enzymes (P-450, prostaglandin metabolism especially) and morphology and response to inducers like TCDD.

2. Liver cells:

The microspectrophotofluorometer will be applied to the problem of hepatocyte heterogeneity in control versus induced animals. The paper given at FASEB will be completed and submitted for publication (method primarily).

The collaborative studies with Dr. Klinger will continue -- studying the developmental patterns of xenobiotic metabolism in various parts of the liver lobule. We will attempt to separate the periportal and centrolobular cells using elutriation, and will investigate metabolism of these cells as a population and as single cells using the microspectrophotofluorometer.

3. Skin cells:

When staff is available, attempts will be made to separate specific types of cells in different layers of the skin and to study variations in xenobiotic/lipid metabolism in these cells. If the microdot/Western blotting methods can be used on skin cells, we will quantify P-450 isozymes in different cells.

Attempts will be made to study distribution and accumulation of chemicals in different cell types of the skin after animal treatment with pollutants like PCBs which are known to accumulate in skin. The hypothesis here is that different cells of the skin (especially those rich in lipids) will accumulate PCBs, etc., differently, and that the accumulated chemical may affect xenobiotic/lipid metabolisms by these cells in different ways.

Taken together, these studies in cells from these different tissues will further delineate the differences in xenobiotic and lipid metabolisms among cell types and give some insights into the different functions of the cells. Correlation of metabolism with accumulation of chemicals (e.g., in the skin with PCBs) will aid understanding of how cell interactions with chemicals can affect accumulation of toxins as well as how toxin accumulation might affect ability of the cell to function or meet the threat of other chemical exposures. Finally, the studies of possible changes which occur in cells during isolation (possibly intracellular proteolysis or adsorption of extracellular proteins?) will further describe the in vitro versus in vivo differences which may be important in extrapolation of our results. Freshly isolated cells seem to be much preferred to cells grown in culture since our data so far point only to quantitative not qualitative differences between enzymes in the isolated cell versus the in vivo situation, whereas with cultured cells, studies have shown both rapid loss as well as differential loss of enzyme systems in comparison with the in vivo situation (e.g., several isozymes of P-450 seem to be lost in cultured cells).

Publications (past 18 months)

Pohl, R.J., Coomes, M.W., Sparks, R.W., and Fouts, J.R.: 7-Ethoxycoumarin O-deethylation activity in viable basal and differentiated keratinocytes isolated from the skin of the hairless mouse. Drug Metab. Dispos. 12: 25-34, 1984.

Devereux, T.D.: Alveolar type II and Clara cells. Isolation and xenobiotic metabolism. Environ. Health Perspect. 56: 95-103, 1984.

Coomes, M.W., Sparks, R.W., and Fouts, J.R.: Oxidation of 7-ethoxycoumarin and conjugation of umbelliferone by intact, viable epidermal cells from the hairless mouse. J. Invest. Dermatol. 82: 598-601, 1984.

Devereux, T.R., Diliberto, J.J., and Fouts, J.R.: Cytochrome P-450 monooxygenase, epoxide hydrolase and flavin monooxygenase activities in Clara cells and alveolar type II cells isolated from rabbit. Cell Biol. Toxicol. 1: 57-65, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 80040-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Developmental Pharmacogenetics of Liver Microsomal Testosterone Hydroxylases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Negishi	Visiting Scientist	LP	NIEHS
Others:	M. Noshiro	Visiting Associate	LP	NIEHS
	B. Burkhart	Biologist	LP	NIEHS
	G. Wong	"Q"	LP	NIEHS
	T. Ichikawa	Visiting Fellow	LP	NIEHS
	K. Kawajiri	Visiting Scientist	LP	NIEHS
	J. Squires	Guest Worker	LP	NIEHS

COOPERATING UNITS (if any)

City of Hope, CA; Laboratory of Genetics, NIEHS

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION

NIEHS/NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

3.5

PROFESSIONAL

2.0

OTHER

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mouse testosterone 16 α - and 15 α -hydroxylases consist of male and female-specific isozymes. Sex-dependent differences of the 16 α - and 15 α -hydroxylase activities in microsomes are a sum of the differential expression of the isozymes in males and females. We have purified the male- and female-specific isozymes of 16 α -hydroxylase and the female-specific 15 α -hydroxylase and prepared specific inhibitory antibodies to the isozymes. The expression of 16 α - and/or 15 α -hydroxylase activities in microsomes differs among inbred strains of mice; the 16 α -hydroxylase is a male-predominant activity in 129/J but not in BALB/cJ mice. The 15 α -hydroxylase activity is 10 times as high in renal microsomes of 129/J females than in those of BALB/cJ females. With the use of specific inhibitory antibodies to the isozymes, the genetic basis of strain differences in the hydroxylase activities was studied with offspring of 129CF1(129/J(♀)XBALB/cJ), C129F1(BALB/cJ(♀)XBALB/cJ), F2(129CF1X129-CF1), 129CF1(♀)X129/J, and 129CF1(♀)XBALB/cJ mice. The low rate of 16 α -hydroxylase activity is due to a repression of the female-specific isozyme in 129/J females, and the repression is inherited as an autosomal recessive trait which is regulated by a single locus (Rip locus). A similar study was carried out for regulation of 15 α -hydroxylase activity in renal microsomes. Its results demonstrated that the female-specific isozyme of 15 α -hydroxylase is expressed 10 times more in 129/J than in BALB/cJ females, and that the higher level of expression is inherited as an autosomal dominant trait which is also regulated by a single locus (RSH locus). The cDNA clones encoding these isozymes of testosterone hydroxylases have been isolated. A genomic DNA library of 129/J was constructed. The genomic DNA clones for the isozyme genes have been isolated from BALB/cJ and C57BL/6J as well as 129/J mice of genomic DNA libraries. By characterization of the cDNA and genomic DNA clones, we intend to elucidate the molecular mechanisms of genetic regulation of the sex-dependent expression of these hydroxylase isozymes in mice.

B. PROJECT DESCRIPTION

DEVELOPMENTAL PHARMACOGENETICS OF LIVER MICROSOMAL TESTOSTERONE
HYDROXYLASES1. Research Project

Nature of problem: A change of cytochrome P-450 composition in a microsomal monooxygenase system can have a pronounced effect on metabolic detoxication and activation of drugs and chemicals. The cytochrome P-450 composition is altered by many factors. Many drugs (e.g., phenobarbital) and chemicals (e.g., polycyclic aromatic hydrocarbons) induce and/or repress forms of cytochrome P-450 resulting in significant alteration of cytochrome P-450 composition and metabolism of exogenous and endogenous compounds. Age, sex and tissues also contribute to the determination of P-450 composition (probably through hormonal and genetic control).

To completely understand the role of cytochrome P-450 in chemical-mediated toxicity, including carcinogenesis, it will be necessary to elucidate the molecular mechanisms for the regulation of cytochrome P-450 gene expression. Recent information (obtained by using cDNA encoding for specific forms of cytochrome P-450) has demonstrated that the induction process is the result of gene expression although the molecular mechanisms of gene expression by chemicals and hormones is virtually unknown. Although cytochrome P-450 tends to exhibit broad substrate specificity toward exogenous compounds, we expect that the cytochrome exhibits higher or even absolute substrate specificity toward endogenous compounds such as steroid hormones. Furthermore, as it is our aim to understand regulation of cytochrome P-450 gene expression by endogenous (biological) compounds such as hormones, we have chosen to use testosterone as a substrate to characterize various forms of cytochrome P-450 and their gene regulation.

Liver microsomal steroid hydroxylase activities are catalyzed by forms of cytochrome P-450. Sex-dependent differences of steroid hydroxylase activities, such as male-specific testosterone 16 α -hydroxylase, female-specific 15 α - or 16 α -hydroxylase, are a reflection of sex-dependent expression for these hydroxylase activities. As expected from their sex dependency, the hydroxylase activities are determined developmentally. The age- and sex-dependent expression of hydroxylases are reversibly or irreversibly fixed. The mechanism of irreversible gene fixation of hydroxylase expression known as "neonatal imprinting by androgen" is an important question to be solved. In inbred mice sex-dependent expression of steroid hydroxylases varies from one strain to another. Testosterone 16 α -hydroxylase activity is high in male 129/J mice but is equally high in female as well as male BALB/cJ and C57BL/6J mice. Testosterone 15 α -hydroxylase activity is high in 129/J females but equally low in female as well as male BALB/cJ mice. By appropriate crosses between these inbred strains, we expect to find single locus regulation of sex-dependent expression of the

hydroxylases. Therefore, naturally occurring genetic variants of mice should provide a suitable experimental system to investigate the molecular mechanism(s) of sex-dependent gene regulation of the testosterone hydroxylases.

Objectives in near term:

We have been investigating mechanisms by which sex-dependent expression of liver microsomal testosterone hydroxylases are regulated in mice. Testosterone 16 α -hydroxylase is a male predominant activity in 129/J mice but not in other strains such as BALB/cJ and C57BL/6J. Testosterone 15 α -hydroxylase is a female predominant activity in 129/J mice but not in BALB/cJ mice. These two liver microsomal hydroxylases were chosen for study. Our objective is to understand the biochemical, genetic and endocrinological basis for sex-dependent regulation of the hydroxylases in inbred strains of mice.

We initially asked how many isozymes of the 16 α - and 15 α -hydroxylase exist in mice and found male- and female-specific isozymes for both 16 α - and 15 α -hydroxylases. Total hydroxylase activity is due to differential expression of the isozymes in male or female mice. Based on the information obtained from biochemical studies (purification of isozymes), our major research is shifting to molecular biological aspects and mouse genetics. With a combination of recombinant DNA techniques, mouse genetics and cell biology, we intend to elucidate the biological mediators which regulate sex-dependent gene expression, the mechanisms by which the mediators transmit information to hydroxylase genes and finally, the mechanisms by which the hydroxylase genes receive the information to modulate expression.

Experimental approach:

Purification and characterization of isozymes:

Using specific testosterone 16 α - and 15 α -hydroxylase activities as the basis for selection of fractions from column chromatography, isozymes of 16 α - and 15 α -hydroxylase were purified from male and female 129/J mice. Octylamino Sepharose 4B, isobutyl Sepharose 4B, DEAE Bio-Gel A, CM-52 and hydroxylapatite were used as packing materials for chromatography. Substrate specificities, regio- and stereospecificities of purified isozymes were studied with reconstituted monooxygenase systems. Polyclonal antibodies were elicited against purified isozymes and immunochemical characterization of isozymes was investigated. Partial amino acid sequencing of isozymes will be studied in collaboration with Dr. J.E. Shively, City of Hope, California.

Isolation and characterization of cDNA clones encoding hydroxylases:

We have used two methods to clone cDNAs encoding hydroxylases. 1) mRNA was enriched by immunoprecipitation of polysomes bearing the relevant mRNA isozymes from total liver polysomes prepared from male or female 129/J mice. The cDNA libraries were constructed from the immunoenriched poly(A⁺)RNAs with expression vector pUC-9. The cDNA encoding hydroxylase isozyme was selected by in situ immunostaining and double colony hybridization to the immunoenriched versus non-enriched cDNAs. 2) The cDNA library was constructed from total liver poly(A⁺)RNAs with GT-11 as cloning vector. This phage cDNA library was screened by an immunochemical method and hybridization to DNA the probe. The isolated cDNAs will be sequenced to determine isozyme sequence and to help analyze their gene structures.

Isolation of genomic DNA clones and analysis of gene structure of hydroxylase:

With the use of cDNAs, genomic DNA libraries of 129/J, BALB/cJ and C57BL/6J mice will be screened to isolate genomic DNA clones containing hydroxylase genes. The genomic DNA library of 129/J will be constructed with EMBL3 as a cloning vector, and the other libraries will be obtained from established laboratories. The isolated genes will be analyzed by means of exon and intron organization and of DNA sequencing of genes, including their 5'- and 3'-flanking regions. The gene structures of different inbred mice will be compared to find whether or not genetic variations arose from mutation of structural genes and to define particular nucleotide sequences responsible for sex-dependent regulation of hydroxylases.

Mouse genetics of 16 α - and 15 α -hydroxylases:

Loci regulating genetic differences in 16 α -hydroxylase activity in 129/J and BALB/cJ mice will be determined with offspring from 129/J X BALB/cJ by using the rate of activity due to the specific isozyme ("C"- or "I"-P-450_{16 α}) and levels of mRNAs of the isozymes. Recombinant inbred strains, NX129 and 9XJ will be used to determine chromosome localization of the loci. Similar studies will be conducted to find a locus by which testosterone 16 α -hydroxylase is regulated between 129/J and BALB/cJ. Since preliminary results indicate that strain differences in 15 α -hydroxylase activity appear to be clear or in kidney than in liver microsomes, kidney was chosen as the tissue to study the genetics of 15 α -hydroxylase.

Recent accomplishments:Purification of female-specific isozyme of testosterone 16 α -hydroxylase:

We have found that mouse testosterone 16 α - and 15 α -hydroxylase consist of male- and female-specific isozymes and have purified the male isozyme of 16 α -hydroxylase and female 15 α -hydroxylase isozyme in the past years.

We also purified and characterized a female-specific isozyme of 16 α -hydroxylase from phenobarbital-treated 129/J female mice. Based on the specific hydroxylase activity in fractions from columns of Octylamino Sepharose 4B, hydroxylapatite, DEAE Bio-Gel A and isobutyl Sepharose 4B, the isozyme was purified to 12.5 nmol P-450/mg protein and to apparent homogeneity based on analysis by SDS polyacrylamide gel electrophoresis. The apparent molecular weight of the isozyme was 54K daltons. The purified isozyme catalyzed testosterone 16 α -hydroxylation at a rate of 8 nmol/min/nmol P-450, and is about 10 times less active than the male-specific isozyme of 16 α -hydroxylase. Both male- and female-specific isozymes catalyzed benzphetamine N-demethylation activity very efficiently. An antibody raised against the purified female isozyme did not inhibit testosterone 16 α -hydroxylase activity reconstituted with male-specific 16 α -hydroxylase but inhibited the activity reconstituted with the homologous antigen. Therefore, male and female 16 α -hydroxylases are immunochemically unrelated isozymes.

Isolation and characterization of cDNA clones for the hydroxylases:

cDNAs encoding the male isozyme of 16 α -hydroxylase were isolated. The largest insert was 1.75 kbp. The cDNA library constructed from immunoenriched poly(A⁺)RNA using pUC-9 as vector was screened by selective hybridization and *in situ* immunostaining. The selected recombinant DNAs were characterized by hybrid selection of mRNA and enrichment of an antibody to the hydroxylase by hybrid protein synthesized in the recombinants. The cDNA p16 α hybridized only with 2.0 kb mRNA, and the amount of the 2.0 kb mRNA was about 10 times more in male than female 129/J mice, indicating pretranscriptional regulation of male predominant expression of this isozyme of 16 α -hydroxylase. Similarly, the cDNA encoding the female isozyme of 15 α -hydroxylase was cloned and characterized. Two different cDNAs representing two alternative transcripts were identified from their restriction maps. The cDNAs were designated as p15-29 and -15, contained 1.6 and 1.5 kbp of insert, respectively, and hybridized only with 2.1 kb mRNA. The amount of the 2.1 kb mRNA was about 6 times higher in liver of female than in male 129/J mice.

We found that female specific mouse 16 α -hydroxylase shared nucleotide sequence homology with phenobarbital-induced rat liver cytochrome P-450_{b/e}. The cDNA clone R17 was used to measure the content of mRNA of the female specific 16 α -hydroxylase.

Isolation and characterization of genomic DNA clones:

We have constructed a genomic DNA library of 129/J containing 2.6×10^6 recombinants by using EMBL3 as a cloning vector. The DNA libraries of BALB/cJ and C57BL/6J were obtained from established laboratories. Southern hybridization of the cDNAs to genomic DNA showed multigene families of male and female 16 α -hydroxylases and female 15 α -hydroxylases. This was confirmed by isolation of three to four different genomic clones for the hydroxylases from the genomic DNA libraries.

Establish mouse genetic models for regulation of hydroxylase:

An inhibiting antibody was used to quantitate the rate of 16α -hydroxylase activity due to the female-specific 16α -hydroxylase and the content of this isozyme in liver microsomes. We found that the rate of activity and content of the hydroxylase were 10 times as high in BALB/cJ as in 129/J females. These differences in the two strains correlated well with the levels of hydroxylase mRNA. The level of hydroxylase mRNA was examined in offspring of 129CF1(129/J \times BALB/J), C129F1(BALB/cJ \times 129/J), F2(129CF1 \times 129CF1), 129CF1 \times 129/J, and 129CF1 \times BALB/cJ. The results showed that the deficiency of the 16α -hydroxylase in 129/J females was inherited as an autosomal recessive trait and regulated by a single locus (Rip locus, regulation of "I"-P-450 16α). Similarly, a single locus regulation of 15α -hydroxylase in mouse kidney was also found. In the same 129/J and BALB/cJ pairs, the deficiency of 15α -hydroxylase in BALB/cJ females was inherited as an autosomal recessive trait and regulated by a single locus (Rsh locus, regulation of steroid 15α -hydroxylase).

2. Plans for Future

The aim of our research is to understand the molecular mechanisms by which sex-dependent expression of male- and female-specific isozymes of testosterone 16α - and 15α -hydroxylases is regulated in mice. In the past two years, we have purified the female and male isozymes of 16α -hydroxylase and the female isozyme of 15α -hydroxylase. We have also isolated cDNA clones and genomic DNA clones encoding these hydroxylase isozymes. The single locus regulation of 16α -hydroxylase and 15α -hydroxylase was established in 129/J and BALB/cJ pairs. The next major question is whether or not these loci represent either cis-acting or transacting genetic elements. Another important question concerns the biological modulator(s) by which sex specific expression of hydroxylase genes are regulated. In order to answer these questions, it is necessary to first understand completely the multiple gene families of each hydroxylase isozyme, and these consist of at least 3 to 6 genes and pseudogenes. Nucleotide sequence analysis of the isolated cDNA and genomic DNA clones will be our most important work in the next year.

Purification of male-specific isozyme of testosterone 15α -hydroxylase:

There also exists a male-specific isozyme of 15α -hydroxylase in mice. This isozyme is not immunochemically related to the female-specific isozyme of 15α -hydroxylase and is induced by pregnenolone 16α -carbonitrile (PCN). This isozyme will be purified and an antibody will be raised against the purified isozyme.

Enzymological study on the isozymes of hydroxylases:

Substrate specificities of the isozymes and K_m and V_{max} of hydroxylase activities with different substrates will be determined with androgens, estrogens, glucocorticoid and mineralcorticoids. This will investigate the biological role of these hydroxylases in the metabolism of steroid hormones and its sex-dependency.

Isolation of cDNA and genomic DNA clones for female isozyme of 16 α -hydroxylase and male isozyme of 15 α -hydroxylase:

We will use GT-11 to construct the cDNA library. The cDNAs encoding these isozymes will be selected by immunochemical methods and selective hybridization. The genomic DNA clones will then be screened by the cDNA as a probe from the genomic DNA library.

DNA sequence analysis of the cDNAs and genomic DNAs encoding the hydroxylases:

In order to elucidate gene structure of hydroxylase isozymes, all cDNAs and genomic DNAs will be sequenced by M13 Sanger's method. This includes 5'-flanking regions of hydroxylase genes.

Chromosome localization of regulatory and structural genes of hydroxylase:

Chromosome localization of structural genes of the hydroxylases will be determined by using somatic cell hybrids and recombinant inbred strains. This part of our work will be done in collaboration with Dr. Skow at this institute. Chromosomal localization of Rip locus and Rsh locus (anticipated to be regulatory genes) will be determined by using recombinant inbred strains NX129 and 9XA.

Hormonal effects on sex-dependent expression of hydroxylases:

Effects of growth hormone, androgens and estrogens are the primary concern in this experiment. Growth hormone-deficient mice, lit/lit and dw/dw will be used for studying growth hormone effects. To investigate the effects of androgens and estrogens, mice will be castrated or ovariectomized and hormonal effects will then be measured on activities and mRNA levels of the hydroxylase isozymes. Alteration of the hydroxylase activities and their mRNA levels by coadministration of drugs such as phenobarbital and PCN with the hormones will also be examined.

3. Publications (past 18 months)

Harada, N. and Negishi, M.: Mouse liver testosterone 15 α -hydroxylase (cytochrome P-450_{15 α}). Purification, regioselectivity, stereospecificity and sex-dependent expression. J. Biol. Chem. 259: 1265-1271, 1984.

Ohyama, T., Nebert, D.W., and Negishi, M.: Isosafrole-induced cytochrome P₂-450 in DBA/2N mouse liver. Characterization and genetic control of induction. J. Biol. Chem. 259: 2675-2682, 1984.

Nebert, D.W. and Negishi, M.: Environmental and genetic factors influencing drug metabolism and toxicity. In Mitchell, J.R. and Horning, M.G. (Eds.): Drug Metabolism and Drug Toxicity. New York, Raven Press, 1984, pp. 137-161.

Harada, N. and Negishi, M.: Mouse liver testosterone 16 α -hydroxylase (cytochrome P-450_{16 α}): Purification, regioselectivity, stereospecificity and immunochemical characterization. J. Biol. Chem. 259: 12285-12290, 1984.

Hankinson, O., Anderson, R.D., Birren, B.W., Sander, F., Negishi, M., and Nebert, D.W.: Mutations affecting the regulation of the cytochrome P₁-450 gene in the mouse Hepa-I cell line. J. Biol. Chem. 260: 1790-1795, 1985.

Harada, N. and Negishi, M.: Sex-dependent expression of mouse testosterone 16 α -hydroxylase (cytochrome P-450_{16 α}): cDNA cloning and pretranslational regulation. Proc. Natl. Acad. Sci. USA 82: 2024-2028, 1985.

Devore, K., Harada, N., and Negishi, M.: Characterization of cytochrome P-450 ("I"-P-450_{16 α}) associated with phenobarbital-induced testosterone 16 α -hydroxylation in mice. Biochemistry, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 80041-02 LP
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Detection and Quantitation of Cytochrome P-450 Isozymes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	R.M. Philpot	Research Chemist LP NIEHS
Others:	B.A. Domin	Staff Fellow LP NIEHS
	P. Bent	Biological Lab. Technician LP NIEHS
COOPERATING UNITS (if any) Department of Biochemistry, Scripps Clinic and Research Foundation, LaJolla, CA; Department of Biochemistry and Drug Metabolism, Hoffmann-LaRoche, Nutley, NJ		
LAB/BRANCH Laboratory of Pharmacology		
SECTION Molecular and Comparative Pharmacology		
INSTITUTE AND LOCATION NIEHS/NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL 1.5	OTHER 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The development of methods for the detection and quantitation of various isozymes of cytochrome P-450 allows for the determination of isozyme distribution and analysis of expression of activities relative to enzyme content. Although it is known that the turnover numbers for some isozymes decrease following isozyme induction, we have now shown that the extent of the decrease depends upon the substrate examined. Treatment of rabbits with TCDD results in a 20-fold increase in the pulmonary content of cytochrome P-450, isozyme 6. The metabolism of two substrates for isozyme 6 also increases, but to different extents, the O-deethylation of 7-ethoxyresorufin increases about 10-fold and the hydroxylation of benzo(a)pyrene increases only about 1.5-fold. Maximum activity with both substrates is obtained by the addition of purified cytochrome P-450 reductase to the microsomal incubations. The induction of isozyme 6 and the differential expression of activity with benzo(a)-pyrene and 7-ethoxyresorufin is also observed with microsomal preparations from alveolar type II cells, Clara cells and pulmonary macrophages isolated from lungs of rabbits treated with TCDD. The effect of TCDD on the pulmonary cytochrome P-450 systems of species other than rabbit has also been examined. Treatment of rats, mice, guinea pigs and hamsters with TCDD induced a form of cytochrome P-450 related immunochemically to isozyme 6. These isozymes are also detected with antibodies to the rat homolog of isozyme 6, P-450. Treatment of all species with TCDD results in increases in the concentrations of two isozymes in liver; the homologs of isozymes 6 and 4. The homolog of isozyme 6 is also detected in placental microsomal fractions from humans exposed to PCBs found in contaminated rice oil.		

B. PROJECT DESCRIPTION

DETECTION AND QUANTITATION OF CYTOCHROME P-4501. Research Project

Nature of problem: Cytochrome P-450 is a family of isozymes that has been incompletely described. The relationships among these isozymes varies considerably. At one extreme are isozymes like 2 and 5 in the rabbit for which no immunochemical, structural or functional similarities have been noted. At the other extreme are genetic variants whose amino acid sequences differ at only a few positions. Some of the problems of detection and quantitation of these isozymes can be solved by "Western blotting" since isozymes can be identified by both immuno-reactivity and molecular weight by this technique. However, it is now clear that there can be multiple isozymes of the same apparent monomeric molecular weight. In such cases specific monoclonal antibodies are required if accurate determinations are to be carried out. In order to determine the microsomal activities catalyzed by a given isozyme and to calculate the extent to which the catalytic potential of the isozyme is expressed, the substrate specificity of the purified isozyme and the microsomal concentration and activities of the isozyme must be known. This requires specific methods for both quantitation and inhibition.

Objectives in near term:

Specific assays for the detection, quantitation and inhibition of rabbit isozyme 5 will be developed. These assays will be based on the selectivity of monoclonal antibodies. All monoclonals will be screened for cross-reactivity with other isozymes and for their usefulness in detection by "Western blotting" and inhibition of activities in microsomal preparations. These assays will be used to determine the responses of isozyme 5 in different tissues to inducers and to calculate the extent to which the catalytic potential of the isozyme is expressed. Monoclonal antibodies will also be used to investigate whether or not isozymes analogous to 5 are present in species other than the rabbit.

Experimental design:

The production of antibodies will be initiated by injection of antigen directly into the mouse spleen. Fusion and culture will be carried out in the normal manner. Primary screening will be done by the ELISA assay using purified antigen. Screening for monoclonals effective in "Western blotting" will be done with hepatic microsomal preparations from rabbits treated with phenobarbital. Inhibitory antibodies will be selected by their effect on the metabolism of 2-aminofluorene in microsomal preparations from rabbit lung. Only antibodies that give greater than 90% inhibition will be used.

Recent accomplishments:

The extent to which the catalytic potential of an isozyme of cytochrome P-450 is expressed in a microsomal preparation can vary markedly with different substrates. In pulmonary microsomes from rabbit, all of the O-deethylation of 7-ethoxyresorufin (ERF) and about 50% of the hydroxylation of benzo(a)pyrene (BP) are catalyzed by isozyme 6. The remainder of the BP hydroxylation, as determined by antibody inhibition, is catalyzed by isozyme 2. Following treatment of rabbits with TCDD, the pulmonary concentration of isozyme 6 increases about 20-fold, ERF metabolism increases about 10-fold, and BP metabolism increases only about 1.5-fold. TCDD has no effect on the concentration of isozyme 2. Results of inhibition studies indicate that BP activity catalyzed by isozyme 6 increases about 2-fold, while that catalyzed by isozyme 2 does not change. Thus, the expression of isozyme 6 activity is about 5-times greater with ERF than with BP in microsomal preparations from treated rabbits, whereas the turnover numbers are only 1/2 (ERF) and 1/10 (BP) those obtained in microsomes from untreated rabbits or in purified systems. The addition of purified cytochrome P-450 reductase to incubations of pulmonary microsomes from treated rabbits increases both ERF and BP activities to the rates expected from the concentrations of isozyme 6 present.

Induction analogous to that observed in rabbit lung is also observed in the lungs of rats, mice, guinea pigs, and hamsters following treatment of these animals with TCDD. In all species the concentration of protein detected with antibodies to isozyme 6 increases markedly after treatment with TCDD. This increase in protein is also detected with antibodies to P-450_C, the rat homolog to isozyme 6. A single protein in lung of all species is also detected with antibodies to isozyme 2 or rat P-450_B. The concentrations of isozyme 2 and P-450_B in liver are increased following treatment of rabbits or rats with phenobarbital. Similar induction is observed in the livers of all species examined; however, phenobarbital has no effect on the concentrations of these isozymes in lung.

The induction of isozyme 6 by TCDD can be detected in type II cells, Clara cells, and pulmonary macrophages isolated from rabbit lung. The extent of induction is greatest in the macrophage, which contains only trace amounts of P-450 isozymes in the non-induced state. Differential expression of ERF and BP activity is observed with all three cell types isolated from lungs of rabbits treated with TCDD.

Placentas from humans exposed to PCBs contain a protein that can be detected by antibodies to cytochrome P-450, isozyme 6. A good correlation between the relative concentration of this protein and the metabolism of ERF and BP is observed. In addition, the BP and ERF activities in human placenta are inhibited by α -naphthoflavone, a potent inhibitor of rabbit isozyme 6.

2. Plans for Future

The roles of isozymes 2 and 6 in the pulmonary metabolism of BP in rabbit lung will be further investigated. The effects of specific inhibitors on metabolite profiles will be compared to the profiles obtained with the purified isozymes. The relationships between the reductase and substrates for isozyme 6 will be investigated. Attempts will be made to identify isozymes analogous to rabbit isozyme 5 in other species.

3. Publications (past 18 months)

Domin, B.A., Serabjit-Singh, C.J., and Philpot, R.M.: Quantitation of rabbit cytochrome P-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase-immunostaining procedure. Anal. Biochem. 136: 390-396, 1984.

Domin, B.A., Serabjit-Singh, C.J., Vanderslice, R.R., Devereux, T.R., Fouts, J.R., Bend, J.R., and Philpot, R.M.: Tissue and cellular differences in the expression of cytochrome P-450 isozymes. In Paton, W., Mitchell, J., and Turner, P. (Eds.): Proc. IUPHAR 9th International Congress of Pharmacology. The Macmillan Press Ltd., 1984, pp. 219-224.

LABORATORY OF PULMONARY PATHOBIOLOGY

THE LABORATORY OF PULMONARY PATHOBIOLOGY

Summary Statement

The Laboratory of Pulmonary Pathobiology (LPP) studies basic mechanisms of pulmonary cell biology and biochemistry as well as mechanisms of toxic cellular injury leading to the development of pulmonary diseases. By investigating normal biological functions as well as their disruption by toxic agents we hope to elucidate pathogenetic mechanisms of disease. The scope of research approaches employed spans from whole animal experiments to cell culture and molecular studies. The Laboratory's research efforts revolve around three major themes: the regulation of cellular differentiation, the mechanisms of neoplastic transformation and the pathobiology of particle and fiber toxicity.

In the following I will highlight a few select research projects in which significant advances have been made during the past year.

The study of Clara cells. The small airways of mammals contain an epithelial cell type, known as the Clara cell, which is characterized by two prominent features: abundant smooth endoplasmic reticulum and membrane-bound electron-dense cytoplasmic granules, presumed to be secretory granules. Studies conducted in a number of laboratories have established that this cell type contains highly active drug metabolizing enzymes. What is not known is the role Clara cells play as secretory cells and the nature and function of the content of their cytoplasmic granules. Another aspect of Clara cell biology, which is of considerable interest, is the proposed role of Clara cells as stem cells in the terminal bronchioles; at present, the extent of their proliferation and differentiation potential is not known. Because Clara cells constitute only a small fraction of the total pulmonary cell population, it is difficult to investigate their biosynthetic and presumed secretory activity in the intact lung. Methods were therefore developed in our Laboratory to isolate Clara cells from protease digests of rabbit lungs with 80 to > 90% purity by the following sequential methods: centrifugal elutriation, density gradient centrifugation and differential adherence. These isolated Clara cells, were shown to synthesize and release a number of proteins which were radiolabeled and analyzed by SDS gel electrophoresis. Two protein bands of 9kd and 180kd apparent molecular weights seemed to be unique to Clara cell cultures, since they were not produced in detectable quantities by purified Type II alveolar cells, pulmonary macrophages or tracheal basal cells. The 9kd protein was also found in serum-free pulmonary lavage fluid obtained from intact rabbit lungs, suggesting that this protein maybe secreted in vivo by Clara cells into the airways. This conclusion was further supported by studies showing that anti-serum prepared against Clara cells reacts with the 9kd protein in pulmonary lavage effluents and that anti-serum made against the 9kd protein purified from pulmonary lavage effluents reacts with 9kd Clara cell secretions produced in vitro. Studies on the cellular and subcellular localization of this 9kd protein and on its possible functional significance are currently under way. Investigations are also in progress to assess the capacity of isolated Clara cells to proliferate and differentiate in vitro as well as in vivo.

Studies on the mechanisms of action of retinoids. Retinoids, vitamin A related compounds, modulate a great number of cell and tissue functions. It has been

shown in several tissue culture systems, such as embryonal carcinoma cell lines, that retinoids can act as inducers of terminal differentiation. Retinoids have also been shown to be antineoplastic agents and it is conceivable that their activity as inducers of differentiation and as antineoplastic agents are related. Since the epithelium of the tracheal-bronchial tract is dependent on vitamin A for its normal muco-ciliary differentiation, our interest in retinoids is two-fold: 1) we wish to determine which biochemical functions, typical of airway epithelium, are regulated by vitamin A, and how; 2) we are interested in exploring the antineoplastic and antipromotional effects of retinoids.

Using tracheal epithelial cell cultures, it was found, that vitamin A is essential for the synthesis of mucin glycoproteins; when vitamin A was removed from the culture media synthesis of mucins ceased. In vitamin A-free tracheal cell cultures, the cells stratified, contained prominent tonofilament bundles, and produced crosslinked envelopes, all features typical of differentiating keratinocytes. Two-dimensional gel electrophoresis of keratin proteins produced by vitamin A-free cultures indicated that several new keratins were synthesized which were absent in vitamin A containing cultures. Thus, it is clear that retinoids play a crucial role in controlling the dual differentiation potential of epithelial stem cells in the conducting airways, namely, their capacity to generate either mucous cells or keratinocytes. Studies are currently underway to identify those keratin genes which are under the direct control of retinoids.

In several in vivo tumor models, notably rodent models of mammary and bladder carcinogenesis, retinoids have been shown to be very effective "chemopreventive agents", and in two-stage skin carcinogenesis, retinoids were found to inhibit the promoting activity of phorbol ester tumor promoters. However, in a number of other tumor models retinoids have failed to inhibit tumor development. We decided to study the antineoplastic effects of retinoids in the rat tracheal epithelial transformation system which was developed in our laboratory. It was found that retinoic acid, as well as several other retinoids, irreversibly inhibit neoplastic transformation of rat tracheal cells in culture. Maximum inhibition (> 90%) was achieved with 10^{-8} M retinoic acid. It appears that the anti-transformation effect of retinoids was due to inhibition of cell replication of transformed cells. Transformed clones isolated during the first 5 wks after carcinogen exposure were found to be far more sensitive to the inhibitory action of retinoic acid than clones isolated at 12 wks of carcinogen exposure. These data indicate that probably only the earliest stage of neoplastic transformation is sensitive to inhibition by retinoids and later stages are insensitive.

In recent years, a great wealth of information has emerged on the role of protein kinases in mitogenic responses and in the regulation of growth of normal, untransformed cells. In many cell systems growth and terminal differentiation are opposing cell functions i.e., cessation of growth is often coupled with terminal differentiation and stimulation of growth is commonly associated with loss of differentiation. We hypothesized that there maybe important commonalities between mechanisms of tumor promoter stimulated responses and mechanisms responsible for the constitutive growth of transformed cells. Since retinoids block the tumor promoting effects of TPA and inhibit neoplastic transformation in several in vivo and in vitro systems, it seemed reasonable to search for a common denominator for these effects. Studies carried out to elucidate mechanisms of retinoid action suggest that retinoic acid either effects the phosphorylation

of specific substrates by protein kinases or inhibits the synthesis of important substrates for phosphorylation by protein kinases. One line of evidence is based on the observation that retinoic acid inhibits the induction by TPA of ornithine decarboxylase which is mediated by protein kinase C, but that neither interferes with TPA binding nor with activation of protein kinase C. Another important observation is that retinoic acid does not inhibit phosphorylation of the EGF receptor which occurs as a result of TPA binding to protein kinase C. Together, these findings suggest that the retinoic acid effects on TPA signal transduction mechanisms must be down-stream of protein kinase C activation. Another line of evidence, pointing in the same direction, is based on the observation that retinoic acid inhibits the growth of Syrian hamster embryo (SHE) cells transfected with v-src DNA but that it stimulates the growth of SHE cells transfected with v-Ha-ras DNA. In the same studies it was shown that retinoic acid does not affect the synthesis of either oncogene products, namely pp⁶⁰src and p²¹ras, respectively. Whether the kinase activity of pp⁶⁰src is altered as a result of retinoic acid treatment, still remains to be determined. These studies may give us important insights into the mechanisms of action of retinoic acid. Furthermore, they will aid in exploring mechanisms of transformation by the two oncogenes v-src and v-Ha-ras.

Studies on mechanisms of neoplastic transformation. One of the intriguing questions in carcinogenesis is how compounds which have no or only weak mutagenic activity, can nevertheless have considerable transforming potential. To address this important question, studies were performed with two classes of human carcinogens, namely asbestos fibers and synthetic steroids such as diethylstilbestrol (DES). Both were inactive in specific locus mutation assays using SHE cells, but caused neoplastic transformation in the same cell culture model with an efficiency similar to that observed with well known mutagenic carcinogens. Both asbestos fibers and DES produced aneuploidy by chromosomal non-disjunction; the asbestos through physical interference with the mitotic spindle or the chromosomes; the DES by disrupting microtubule organization. Asbestos-transformed cell lines showed a nonrandom chromosome change, namely trisomy of chromosome 11, suggesting that changes in gene dosage may be an important step in neoplastic transformation of SHE cells by asbestos.

Other studies were concerned with the molecular basis of the multi-step process of neoplastic transformation. These studies demonstrated that multiple oncogenes are required for neoplastic transformation of normal SHE cells. While transfection with polyoma virus DNA (which contains three different oncogenes) caused rapid neoplastic transformation with high frequency, transfection with v-Ha-ras or v-myc alone, did not transform normal SHE cells. V-src DNA transformed SHE cells with low frequency and the few v-src transformed clones which produced tumors upon inoculation into nude mice had long latency periods. This suggests that more than one step may be involved in transformation of SHE cells with v-src DNA. The expression of v-src RNA initially appeared to be inhibited in transfected SHE cells, but was detected in tumor lines derived from v-src transfected cells. Cotransfection of normal SHE cells with v-Ha-ras plus v-myc resulted in neoplastic transformation. The tumors derived from v-Ha-ras plus v-myc cotransfected SHE cells appeared to be monoclonal and showed a nonrandom chromosome change, monosomy of chromosome 15. This indicates that at least three steps may be involved in neoplastic transformation of normal SHE cells, depending on the oncogenes involved and that loss of a suppressor gene may be one of the steps. The involvement of suppressor genes or factors in transformation

of SHE cells is also supported by studies with cell-cell hybrids between normal, preneoplastic and neoplastic SHE cells. When normal cells or some preneoplastic cells, were fused with neoplastic cells, the neoplastic phenotype, was suppressed, while fusion products between two neoplastic cells were always neoplastic. Studies of transfection of preneoplastic SHE cells induced by either DES or asbestos with various oncogenes further support the concept of multistage neoplastic transformation and showed that with some preneoplastic cell lines, conversion from the preneoplastic to the neoplastic stage requires at least two steps. Current studies are concentrating on mechanisms of regulation of oncogene expression, the importance of gene dosage in transformation and the role of suppressor genes or factors in the transformation process.

Studies on mechanisms of particle and fiber toxicity. Inhalation exposure to certain particles and fibers such as asbestos not only increases the risk to develop lung cancer, it also causes fibrotic lung disease. The pathogenesis of pulmonary fibrosis, which in severe cases is a life threatening disease, is complex and only incompletely understood. The studies on asbestos toxicity were focused on the early cellular and anatomic derangements as well as early biochemical events triggered by the interaction of fibers and pulmonary cells. The purposes of the morphological studies were: to determine which tissue compartments and anatomic sites are the targets for inhaled fibers, to determine the features characteristic of the early response and to map the time course of the crucial cellular events. These studies have identified the proximal alveolar duct bifurcations as well as the terminal bronchioles as the most important targets in the lung. Within 24 hrs of a brief inhalation exposure to chrysotile asbestos, DNA synthesis in epithelial cells of the terminal bronchioles and the alveolar duct bifurcations markedly increased; this was followed by an increased DNA-synthesis in interstitial cells at 48 hrs. Morphometric studies showed that the increase in DNA-synthesis was accompanied by an increase in cellular and extracellular tissue mass in the two anatomical locations, involving both epithelial cells as well as interstitial fibroblasts. The evidence to date indicates that the early tissue responses in the region of the alveolar ducts lead to localized pulmonary fibrosis which can be detected as early as one month after a single 5 hr inhalation exposure to asbestos.

Other studies are concerned with the role of pulmonary macrophages in the pathogenesis of alveolar fibrosis. Macrophages were shown to accumulate within hours after exposure to asbestos fibers at the main point of fiber deposition, namely the alveolar duct bifurcations. Attempts were made to identify the signal which attracts these macrophages to the point of fiber deposition. Several lines of evidence indicated that fibers activate a complement dependent chemotactic factor in the alveolar lining layer which results in migration of macrophages to the site of deposition. It was further shown that mice genetically deficient in the fifth component of complement, have a markedly reduced macrophage response to inhaled fibers.

One of the major functions of macrophages is to remove foreign materials, such as particles deposited in the lungs. However, during this process of phagocytosis, macrophages release a variety of substances, some of which are cytotoxic. Studies showed that during the binding of fibers to the macrophage cytoplasmic membrane as well as during the process of phagocytosis, a variety of arachidonic acid metabolites, including prostaglandins and leukotrienes, are released. These metabolites are potent mediators of inflammation and may contribute signi-

ficantly to the development of pulmonary fibrosis. Studies are currently underway to determine the mechanism by which the interstitial fibroblasts are stimulated to proliferate in asbestos exposed lungs.

Also under investigation are the biochemical mechanisms involved in the binding of particles and fibers to alveolar macrophage membranes as well as in the process of phagocytosis. Studies with several lectins have shown that binding and subsequent uptake of positively charged particles are mediated by negatively charged, cell surface sialic acid groups. These studies will not only elucidate the mechanisms involved in particle binding and uptake by pulmonary macrophages, they can also provide us with important clues regarding those features of particle and fiber surfaces which are crucial for their toxicity.

Another aspect of particle toxicity of the lungs which is under investigation in our laboratory is concerned with the toxicity of silica dust. Silica was found to cause accumulation of large amounts of phospholipids in the lungs of exposed animals; a single exposure resulted in a > 15-fold increase above normal within one month after exposure. Most of the accumulated lipids were shown to be surfactant phospholipids. Further studies suggested that the major cause for the accumulation of surfactant phospholipids was an increased rate of surfactant synthesis, which was elevated by > 4-fold. The rate of transfer from the intra to the extra-cellular pool (secretion) and the rate of removal from the extra-cellular pool were only slightly increased, indicating that neither surfactant secretion, nor the mechanisms responsible for removal of surfactant from the lungs were able to cope with the increased synthesis. Preliminary cellular studies suggested that the number of Type II alveolar cells (the producers of surfactant) as well as the size of Type II cells was increased. In the future, studies will be undertaken to determine the mechanisms by which silica particles stimulate surfactant synthesis by Type II alveolar cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-25001-08 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Role of Mutagenesis in Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J. C. Barrett	Research Chemist	LPP, NIEHS
Others:	M. Oshimura	Expert	LPP, NIEHS
	N. Tanaka	Visiting Fellow	LPP, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pulmonary Pathobiology

SECTION

Environmental Carcinogenesis Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.0

PROFESSIONAL

2.0

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type Do not exceed the space provided)

Most chemical carcinogens induce DNA damage and are mutagenic at specific genetic loci; however, certain carcinogens (including the human carcinogens diethylstilbestrol, asbestos, arsenicals and benzene) usually do not induce gene mutations. We have examined the activity of these chemicals, proposed as carcinogenic but not mutagenic, to induce morphological transformation, gene mutations and chromosome mutations in Syrian hamster embryo cells in culture. We have reported previously that diethylstilbestrol (DES) induces transformation in the absence of mutations at specific genetic loci. Furthermore, we have proposed that the mechanism of action of DES is related to its ability to induce numerical chromosome changes, i.e., aneuploidy. We have now shown that DES has colcemid-like activity in that it disrupts microtubule organization. Inhibition of polymerization of spindle microtubules is therefore a possible mechanism for DES-induced aneuploidy and possibly cell transformation according to our hypothesis. This hypothesis is further supported by our observations that colcemid and vincristine sulfate, two well-known inducers of aneuploidy, are also inducers of morphological transformation. The mechanism of another important human carcinogen, asbestos, was also examined. The ability of asbestos and other mineral fibers to induce cell transformation was observed to depend on fiber dimension similar to the results found *in vivo* in studies on mesothelioma induction. We have proposed that asbestos induces cell transformation due to its ability to induce chromosomal changes in the treated cells. We recently found that asbestos fibers induce anaphase abnormalities indicating that a direct physical interaction of the fibers with chromosome occurs during mitosis. In the asbestos-induced cell lines a nonrandom chromosome change, trisomy of chromosome 11, was found. These results further support our hypothesis that the mechanism of asbestos-induced transformation is due to a chromosomal mutation. Thus, our results suggest an important role for carcinogen-induced aneuploidy in carcinogenesis.

PROJECT DESCRIPTION

A significant number of known chemical carcinogens have been shown to be activated to reactive, electrophilic derivatives which directly interact with DNA and cause mutations at specific genetic loci. This has led to the general hypothesis that carcinogenesis results from gene mutations generated by carcinogens damaging DNA. This hypothesis is supported by several lines of evidence and is generally accepted as the mechanism of action of many chemical carcinogens. However, some chemical carcinogens are inactive in short-term tests for DNA damage or specific locus mutations which may indicate alternative mechanisms for chemically-induced cancers. While these chemicals may be the exceptions to the correlation between carcinogenesis and mutagenesis and limited in number, many of these substances are known human carcinogens (e.g., asbestos, benzene, arsenicals, and diethylstilbestrol) and therefore, a better understanding of their mechanism of action is highly significant to carcinogenesis in humans.

Our approach to understanding the role of mutagenesis in carcinogenesis has been to develop an experimental model which can detect different stages in the neoplastic development of cells in culture and to compare the abilities of chemicals to induce neoplastic transformation and different types of mutational changes concomitantly in the same cells. The objectives of this research are:

(1) To determine the activity in the Syrian hamster embryo cell transformation assay of known human and/or animal carcinogens with emphasis on chemicals which are inactive in certain mutational assays such as the Ames test.

(2) To correlate the ability of chemicals to induce cell transformation, gene mutations, and chromosome mutations.

(3) To understand the mechanism of action of chemicals which do not induce gene mutations but induce cell transformation.

(4) To determine the mechanism by which tumor promoters enhance or induce cell transformation.

The experimental system for these studies is the Syrian hamster embryo (SHE) cell transformation assay. Early passage, normal, diploid SHE cells, when exposed to chemical carcinogens, undergo a progressive series of changes resulting in the neoplastic transformation of the cells. Various phenotypic markers can be used to quantitate early, preneoplastic changes (e.g., morphological transformation) and later stages associated with the neoplastic conversion of the cells (e.g., anchorage-independent growth). In this project, the mechanism of the initial stage in this neoplastic progression is studied by comparing the ability of different chemicals to induce morphological transformation and mutations in the same cells. Three types of mutational activity are measured: (1) gene mutations at two specific genetic loci (HPRT and Na⁺/K⁺ ATPase); (2) chromosome aberrations (breaks, fragments, and rearrangements); and (3) aneuploidy, i.e., numerical chromosome aberrations. DNA damage in the SHE cells is also measured by an unscheduled DNA synthesis (UDS) assay.

We have reported that diethylstilbestrol (DES) induced morphological and neoplastic transformation of Syrian hamster embryo cells (SHE) in the absence of detectable gene mutations at two genetic loci. We have found that although a natural estrogen, 17 β -estradiol, is similar to DES in its transforming activity, transforming activity and estrogenic activity do not correlate for a number of DES-related compounds. Furthermore, SHE cells have no detectable estrogen receptors and are not stimulated to grow by estrogens. Based on these observations, the estrogenic activity of these chemicals does not appear to be involved in their transforming ability. Rather we propose that DES and 17 β -estradiol have the ability to induce heritable alterations in cells which lead to the tumorigenic state. This transforming activity in conjunction with estrogen-stimulated cell proliferation may, in combination, result in hormonal carcinogenesis.

In an attempt to understand the mechanism of DES-induced cell transformation, we have examined the ability of DES to induce a variety of genetic changes in the Syrian hamster embryo cells. We have shown that DES induces cell transformation without causing gene mutations, unscheduled DNA synthesis, sister chromatid exchanges, or structural chromosome aberrations. However, DES is an effective inducer of aneuploidy, possibly via nondisjunction. Both chromosome losses and gains are induced by DES doses which are nontoxic but transforming. The importance of aneuploidy induction in DES-induced transformation is suggested from the findings that DES induces aneuploidy and cell transformation with parallel dose-response curves; aneuploidy induction correlates with the ability of DES-related compounds to induce cell transformation; cell cycle specificities of aneuploidy induction and cell transformation by DES indicate that cells in mitosis are most sensitive; and neoplastic cell lines induced by DES are aneuploid with a near-diploid modal chromosome number.

In collaboration with Dr. Robert Tucker of Johns Hopkins University, we have examined the effects of DES on microtubule organization in Syrian hamster embryo cells. We found that concentrations of DES which cause aneuploidy also produce abnormal or arrested mitotic spindles by inhibiting microtubule polymerization from the centrosome. These studies provide a biochemical mechanism by which DES may disrupt the spindle apparatus and produce aneuploidy that results in cell transformation.

17 β -Estradiol (E₂) is similar to DES in its ability to induce transformation although slightly higher doses are required. Like DES, E₂ also fails to induce gene mutations but induces nondisjunction. The dose-response curves for cell transformation and aneuploidy induction by E₂ are similar. These results indicate that both estrogens induce cell transformation by causing numerical chromosome aberrations.

Both DES and E₂ at high doses arrest cells in mitosis suggesting they are mitotic inhibitors. Therefore, it was of interest to determine whether cell transformation could be induced by other mitotic inhibitors, such as colcemid and vincristine sulfate, which are also known inducers of aneuploidy. Treatment of the cells with colcemid results in morphological and neoplastic transformation of the cells. Cell transformation is induced with doses which are non-cytotoxic

and below the concentration necessary to cause mitotic inhibition of the cells. Higher doses of colcemid result in mitotic inhibition of the cells and a significant loss of colony forming ability, but no increase in morphological transformation. Treatment of the cells with transforming doses of colcemid do not result in any measurable induction of gene mutations or structural chromosome aberrations; however, numerical chromosome changes are observed. The dose-response curves for colcemid-induced morphological transformation and aneuploidy induction are similar. The shape of the dose-response curves for DES- and colcemid-induced transformation and aneuploidy are also similar. Maximum induction of both endpoints occurs at doses of DES or colcemid which do not increase the mitotic index. We interpret this finding to indicate that aneuploidy occurs at doses which cause chromosome segregation errors without mitotic inhibition.

Vincristine sulfate also induces morphological transformation and aneuploidy in Syrian hamster embryo cells. It resembles colcemid in its action except that nondisjunction occurs primarily at doses which also increase the mitotic index of the treated cultures. These results further support our hypothesis that cell transformation can be induced by numerical chromosome changes. However, the biochemical mechanisms by which mitotic inhibitors cause aneuploidy, and thereby transformation, may be different.

Asbestos and other mineral fibers are carcinogenic in animals and in man. In terms of induction of mesotheliomas in rats following intrapleural injection, the physical rather than the chemical nature of the fibers is related to the carcinogenic potential of diverse mineral dusts. Asbestos and other fibers are often cited as examples of non-mutagenic carcinogens. We were interested in whether asbestos and other mineral dusts could induce cell transformation since other effects on cells in culture, including toxicity and chromosomal damage, had been reported. Therefore, we examined whether these substances were active in the Syrian hamster embryo system.

We observed that different forms of asbestos, nonfibrous mineral dusts, and glass fibers of varying dimensions induced a dose dependent transformation of Syrian hamster embryo cells. Our results indicated that fiber dimension was very important in the transforming activity of these mineral dusts. The relative potencies of mineral dusts in the induction of cell transformation in vitro is similar to their potencies in the induction of mesotheliomas in vivo. Thus, this system provides a unique model for studying the mechanism of mineral fiber tumorigenesis.

We have examined the mechanism of asbestos-induced transformation and in particular the role of genetic events in this process. We did not detect any mutagenic activity of transforming doses of asbestos when we measured the induction of mutations at two genetic loci; however, we observed that asbestos and other mineral fibers are inducers of chromosomal mutations. We have proposed that this type of genetic change as a possible mechanism for asbestos-induced transformation. Asbestos fibers are taken up by the cells within 24 hours after treatment by endocytosis; the intracellular fibers accumulate around the perinuclear region of the cells by 24-48 hours after exposure.

When the cells undergo mitosis, the physical presence of the fibers results in interference with chromosome segregation. Analysis of anaphases in chrysotile-exposed cells reveals a large increase in the number of cells with anaphase abnormalities including lagging chromosomes, bridges and sticky chromosomes. Asbestos fibers are observed in the mitotic cells and appear, in some cases, to interact directly with the chromosomes. From these studies we propose that the physical interaction of the asbestos fibers with the chromosomes or structural proteins of the spindle apparatus causes mis-segregation of chromosomes during mitosis resulting in aneuploidy. These findings provide a mechanism, at the chromosomal level, by which asbestos and other mineral fibers might induce cell transformation and cancer.

This hypothesis is supported by two additional observations. First, the abilities of different mineral dusts to induce cell transformation correlates very well with their abilities to induce aneuploidy. For example, reducing the length of glass fibers reduces their ability to induce transformation and aneuploidy in parallel. Second, immortal cell lines induced by asbestos show numerical chromosome changes, particularly trisomy of chromosome 11 (in 75% of the lines examined to date). Thus, this is an early, nonrandom change in the neoplastic progression of these cells in culture. These studies indicate that a genetic mechanism at the chromosomal level is involved in asbestos carcinogenicity.

Our results suggest that genetic mechanisms may be involved in the carcinogenic potential of chemicals previously considered to be non-mutagenic. There is good evidence from our studies that certain hormones as well as mineral dusts have mutagenic activity at the chromosomal level. Since this genetic activity is primarily aneuploidy induction, it is not detected by many mutagenic assays, possibly accounting for the putative non-mutagenicity of these agents in other studies. We also have data indicating that other "non-mutagenic" carcinogens, such as benzene and arsenic, cause cell transformation by chromosomal damage, which indicates that this type of genetic event is important for a number of carcinogens.

Future studies will examine the mechanism of action of benzene, sodium bisulfite, diethylhexylphthalate and 2,3,7,8-tetrachlorodibenzodioxin. Preliminary evidence exists that at least the first three compounds are active in the cell transformation assay. Since these are suggested as inactive gene mutagens, we hope to extend our observations on the role of chromosome mutagens in cell transformation and carcinogenicity. In addition, transformed cells will be examined for specific chromosome changes and these will be correlated with molecular studies of the transformed phenotypes of these cells. The mechanism by which asbestos and mineral fibers and other chemicals induce aneuploidy will be examined. Attempts to develop genetic assays for measuring the ability of a chemical to induce aneuploidy and gene amplification will be made.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-25020-03 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Regulation of the Pulmonary Surfactant System and its Modification by Toxic Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	G. E. R. Hook	Research Chemist	LPP, NIEHS
Others:	L. B. Gilmore	Biologist	LPP, NIEHS
	B. A. Miller	Graduate Student	LPP, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pulmonary Pathobiology

SECTION

Biochemical Pathology Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.5

PROFESSIONAL

0.5

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Pulmonary surfactant is synthesized and secreted by alveolar Type II cells. It is a complex mixture of lipids and proteins that prevents collapse of the alveoli and distal airways at low lung volumes. The pulmonary surfactant system is affected by numerous chemical and particulate toxicants through unknown mechanisms. Silica dust, when administered by either intratracheal injection or by inhalation, causes massive increases in the surfactant content of the lungs. The objectives of this work are to elucidate the mechanisms through which the pulmonary surfactant system is stimulated by silica and the processes responsible for the accumulation of phospholipids in the lungs. Twenty eight days after a single intratracheal injection of silica into the lungs of rats (200mg/Kg) the phospholipid content of the lungs increased from 29.6 ± 4.4 to 476.1 ± 129.2 mg. Intracellular and extracellular surfactant pools accounted for 59.1 and 24.6% of this increase in phospholipids, respectively. These silica-induced increases in phospholipids of the intra- and extracellular surfactant pools were both dose- and time-dependent. Although the phospholipid content increased, the phospholipid composition of pulmonary surfactant did not change, in response to silica. Silica (10mg/Kg) increased surfactant phospholipid biosynthesis in the lungs of rats approximately 4-fold. Phospholipid fluxes between the intra- and extracellular pools increased from 115 μ g/hr to 437 μ g/hr. The turnover time for phospholipid in the intracellular pool was increased 3.5-fold from 13 hr to 45.4 hr but the turnover time for the extracellular pool increased only marginally from 10.4 hr to 13.9 hr. No evidence for recycling of surfactant was found. These data indicate that the expansion of the intra- and extracellular pools of surfactant phospholipids was due to increases in the biosynthesis of phospholipids and alterations in the kinetic relationship between the two pools.

PROJECT DESCRIPTION

The mechanisms through which silica stimulates the pulmonary surfactant system are not known although, potentially, such mechanisms could prove extremely important in understanding the processes through which the surfactant system is regulated and controlled. The objectives of this study are to elucidate the mechanisms through which silica stimulates surfactant biosynthesis and secretion in the lungs. Twenty-eight days after a single intratracheal injection of silica into the lungs of rats (200mg/Kg) the DNA and protein content of the lungs increased 3.4-fold and 3.5-fold, respectively. However, during this same period the phospholipid content of the lungs increased 16.1-fold from 29.6 ± 4.4 mg to 476 ± 129 mg. Subcellular fractionation of the lungs by using differential centrifugation revealed that most of this increase in phospholipids was due to increases in the phospholipid contents of the intra- and extracellular pools of pulmonary surfactant. The intra- and extracellular pools accounted for 59.1 and 24.6% of the total lung increase in phospholipids, respectively. The intracellular pool of surfactant increased 80-fold from 1.18 ± 0.65 to 144.9 ± 53.8 mg phospholipid per pair of lungs and the extracellular pool increased 22-fold from 1.17 ± 0.04 to 25.1 ± 7.1 mg phospholipid per pair of lungs. The phospholipid composition of the intra- and extracellular pools of surfactant did not change excepting for phosphatidylinositol which increased almost 2-fold from approximately 3% to 6% in both pools. Phosphatidylcholine was the major phospholipid accounting for approximately 83% of the total phospholipid. Phosphatidylcholine consisted of approximately 38% unsaturated and 62% disaturated phosphatidylcholines indicating a typical composition for pulmonary surfactant. Phosphatidylglycerol accounted for approximately 5% of the total phospholipid in both pools. These data indicated that the phospholipid response of the lungs to intratracheally instilled silica was confined primarily to the surfactant system but compositional changes did not occur in spite of very large increases in the surfactant pools.

Increases in phospholipids could arise from a variety of effects of silica on the dynamics of the surfactant system. The effects of silica on biosynthesis of pulmonary surfactant were investigated by measuring the incorporation of ^{14}C -choline into phosphatidylcholine. The kinetics of the system was investigated by measuring the transfer of radiolabelled phosphatidylcholine between the intracellular and extracellular pools of surfactant and interpreted according to a two compartment model in which we assume the existence of a precursor/product relationship between the two pools. Silica injected intratracheally (10 mg/Kg) into the lungs of rats increased the biosynthesis of surfactant phosphatidylcholine approximately 4-fold after three days. The flux of phosphatidylcholine between the intracellular and extracellular pools of surfactant increased from $115 \mu\text{g/hr/pair of lungs}$ to $438 \mu\text{g/hr/pair of lungs}$. The turnover time for phosphatidylcholine in the intracellular pool increased from 13 hr to 45 hr. In the extracellular pool the turnover time for phosphatidylcholine increased from 10.4 hr to 13.9 hr. Recycling of surfactant phosphatidyl choline was not detected in either treated or control lungs. Removal of phospholipids from the extracellular pool of surfactant by alveolar macrophages was also investigated with negative results. Alveolar macrophages do not appear to be active in the removal of surfactant phospholipids from the alveoli of control lungs nor

silica-treated lungs in spite of large increases in the numbers of free and actively phagocytizing cells. These data indicate that expansion of the intracellular and extracellular pools of surfactant is due to the stimulatory effects of silica on phospholipid biosynthesis and the kinetic relationship between the two pools.

The effects of silica appear to be confined primarily to the surfactant system and, therefore, the major alterations must be associated with the alveolar Type II cells. Our major objective in the next year will be to determine the effects of silica on the Type II cells of the lungs. To account for the expansion of the intracellular pool of surfactant, silica must produce either more Type II cells or Type II cells that contain more surfactant and synthesize surfactant at higher than normal rates. Surfactant biosynthesis will be examined in Type II cell isolated from the lungs of silica-treated and control rats to determine if the major alterations are associated with the subcellular regulation of the phospholipid pathway. The direct effects of silica on Type II cells isolated from the lungs will also be examined as a means of probing the mechanisms responsible for the observed alterations in the surfactant system. Based upon statistically highly significant correlations between the surfactant pools and extracellular soluble proteins in the lungs of silica-treated rats we hypothesize the existence of soluble factors that stimulate the production of surfactant in Type II cells.

The path that leads from inhalation of silica dust to silicosis is obscure. Fibrogenesis is a mandatory step but events leading to and subsequent to fibrogenesis are not known. Early events could be especially significant because reversal of the disease may be possible only in the initial stages. One of the distinctive early events following exposure of animals to silica dust is a marked stimulation of phospholipid biosynthesis and accumulation of phospholipids within the lungs. The reasons for this stimulation are not known although it has been speculated that increased phospholipids could represent a defensive action of the lungs against the cytotoxic and fibrogenic effects of the silica. Regardless of reasons, there seems little doubt that stimulation of phospholipid biosynthesis is an event that occurs very early after exposure to silica and as such could prove of primary significance in understanding the processes that ultimately contribute to the disease state.

PUBLICATIONS

Dethloff, L. A., Gilmore L. B., Brody, A. R. and Hook, G. E. R.: Induction of intra- and extracellular phospholipids in the lungs of rats exposed to silica. Biochem J. In Press, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 25021-02 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Differentiation and Differentiative Functions of Tracheal Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. M. Jetten	Sen. Staff Fellow	LPP, NIEHS
Others:	J. Rearick	Staff Fellow	LPP, NIEHS
	H. Smits	Visiting Fellow	LPP, NIEHS
	M. E. Porter	Biol. Lab. Tech.	LPP, NIEHS
	M. Deas	Chemist	LPP, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pulmonary Pathobiology

SECTION

Cell Biology Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

4.0

PROFESSIONAL

2.5

OTHER

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.)

Rabbit tracheal epithelial cells grown on fibronectin-albumin-vitrogen coated dishes undergo squamous cell differentiation when reaching high density. In the last stage of this terminal differentiation cells undergo cornification. We have established several factors that regulate this differentiation and several biochemical parameters that can function as markers for this differentiation. We have shown that the addition of serum, the omission of EGF from the medium, high density and high calcium concentrations can promote differentiation whereas retinoids inhibit squamous cell differentiation. We have shown that the induction of squamous cell differentiation is accompanied by the synthesis of several new proteins and is regulated by retinoids. Undifferentiated rabbit tracheal epithelial cells express seven major keratins and a new 48 kd keratin is expressed when cells are induced to squamous cell differentiation. In vitro translation experiments have indicated that the synthesis of this 48 kd keratin is regulated at the level of mRNA. Cells undergoing squamous cell differentiation are also induced to synthesize transglutaminase activity. This transglutaminase has been identified as the "epidermal" transglutaminase on basis of its immunoreactivity with a monoclonal antibody against human transglutaminase from human keratinocytes. When cells are grown on collagen gel and in the presence of retinoids cells containing secretory granules were observed by electron microscopy and we have shown via biochemical techniques that these cultures produce mucin glycoproteins. The presence of retinoids appears to be important since in its absence cells don't contain secretory granules and undergo squamous cell differentiation. Studies are underway to determine how differentiation is regulated at the molecular level.

PROJECT DESCRIPTION

The basic structure of the lining of the trachea in situ is a pseudostratified, columnar epithelium, which has been shown ultrastructurally to contain a diverse population of cell types. Although, the exact pathways of differentiation are not yet fully established, it is believed that the differentiated cells develop either directly from the basal cells or via mucous-secreting cells. Under various conditions such as mechanical or toxic injury or vitamin A-deficiency, the basal and/or mucous-secreting cells express a differentiation potential in the form of squamous metaplasia and keratinization. Our objectives are to understand the mechanism by which differentiation of the epithelium of the respiratory tract is regulated.

More specifically:

- (1) To determine what biochemical changes take place during differentiation and at what level they are regulated.
- (2) Is there a relation*between control of proliferation and differentiation?
- (3) What is the molecular mechanism that determines the two pathways of differentiation.

Rabbit tracheal epithelial cells grown on fibronectin-albumin-vitrogen coated dishes undergo squamous cell differentiation when reaching high density. In our studies we have been focussing on the formation of cross-linked envelopes, transglutaminase activity, keratin synthesis and production of cell surface components that are likely to play a role during this differentiation and could function as differentiative markers. We have shown that several factors such as serum, high Ca^{2+} and high density stimulate the squamous phenotype and cornification whereas retinoids have an inhibitory effect.

Induction of transglutaminase activity during squamous cell differentiation

Transglutaminases are a group of enzymes that covalently link peptide-bound glutamine to primary amines. The "epidermal" transglutaminase appears to play a role in the formation of cross-linked envelopes and is likely to play a role during squamous cell differentiation of rabbit tracheal epithelial cells as well. We have characterized the synthesis of transglutaminase activity in these cells and studied its synthesis in relation to the formation of cross-linked envelopes. We have shown that undifferentiated cells produce very low levels of transglutaminase whereas cells at high density produce twenty times more of this enzyme. The induction of this enzyme activity is accompanied by the formation of cross-linked envelopes. All transglutaminase activity is associated with the particulate fraction and can be solubilized by Triton X-100. A monoclonal antibody against "epidermal" transglutaminase precipitates all transglutaminase activity which migrates as a band with a molecular weight of 92 kd on a polyacrylamide gel. Conditions that stimulate squamous cell differentiation, such as the addition of serum or the omission of EGF, induce transglutaminase activity whereas retinoids inhibit the induction of this enzyme. These results show that the induction of transglutaminase activity correlate with the induction of squamous cell differentiation.

Changes in intermediate filaments during squamous cell differentiation

Epithelial cells of the rabbit trachea in primary culture express different keratin patterns depending on the growth stage and culture conditions. Cells in the exponential growth phase express a set of seven major keratins with molecular weights of 58, 56, 54, 50, 48, 43 and 40 kd. These keratins were identified by their relative mobility in two-dimensional polyacrylamide gels and by their reactivity with two monoclonal antibodies (AE1 and AE3) directed against human epidermal keratins. A pronounced change in cellular morphology is observed at the end of the exponential growth phase. At this time cells undergo squamous cell differentiation and express two additional keratins with molecular weights of 48 kd and 54 kd (designated as the 48' kd and the 54' kd keratins). Moreover, the relative expression of the 56 kd keratin is enhanced. In cells grown in the presence of fetal bovine serum or high calcium concentrations the same changes in phenotype and keratin expression are observed. Treatment of cells in the exponential growth phase with retinoic acid prevents the induction of the squamous phenotype and cornification and prevents the alteration in keratin expression except that the 54' kd keratin is still expressed. The action of retinoic acid is reversible: upon removal of retinoic acid cells become squamous and express the 48' kd keratin. We have shown via in vitro translation that the expression of the 48' kd keratin is regulated at the level of mRNA. Our results indicate that the expression of the 48' kd keratin correlates with the induction of the squamous cell phenotype and is regulated by retinoic acid.

We have shown that induction of squamous cell differentiation is accompanied by the synthesis of new proteins and is regulated by retinoids. We like to identify the genes that are differentially expressed and controlled by retinoids. To determine this we are establishing a cDNA library from the differentiated cells and via differential hybridization will select for the cDNA clones of retinoid-controlled genes and these that are expressed preferentially in differentiated cells. We will identify these cDNA clones and characterize their products. The genes will be analyzed for their DNase I hypersensitivity, methylation and interaction with retinoids.

We have indications that the regulation of differentiation is closely related to the regulation of proliferation. When cells reach confluency their proliferation ceases, cells start to accumulate in G₁ and cells become committed to terminal squamous cell differentiation. Inhibition of cell growth by the addition of serum or TGF β also stimulates cellular differentiation. EGF appears an important factor in the regulation of cellular proliferation, omission of EGF from the medium inhibits proliferation and induces differentiation. We have hypothesized that when cells become committed to terminal differentiation they are no longer responding to triggers such as EGF that induce proliferation. In this case the EGF receptor may not longer be functional. This could be overcome by introducing an EGF-receptor that acts in a constitutive manner such as the erb-B product. This hypothesis is testable, we are in the process of testing the activity of the EGF-receptor in differentiated and undifferentiated cells and are isolating cells transfected with erb-B that are no longer responsive to triggers that induce differentiation.

Regulation of the synthesis of cellular and secreted glycoconjugates

The substratum has been shown in various systems to be important in inducing proper differentiation. Cells plated on collagen gels in the absence of vitamin A undergo stratification and cornification whereas in the presence of vitamin A cells become cuboidal and contain secretory granules. To gain more information on the role of the cell-substratum interactions in differentiation and the regulation of the synthesis of the secretory products, we have begun to identify the cell surface components and secretory products. To identify and quantitate these molecules cells were metabolically labeled with ^3H -glucosamine and/or ^{35}S -sulfate.

Mucous glycoproteins were isolated, characterized and quantitated by methods identical to those used by us in studying mucous glycoproteins from hamster tracheal epithelial cells. Radiolabeled proteoglycans were isolated and characterized by methods developed by V.C. Hascall which include quantitative extraction in a denaturing buffer, separation by ion exchange chromatography and characterization by gel filtration and by chemical and enzymatic degradative procedures specific for various proteoglycan classes. Sulfatides were estimated by measuring the amount of ^{35}S radioactivity remaining in the lower phase of a Folch extraction of cellular radiolabeled material.

RbTE cells grown on collagen gel in the presence of retinoic acid (RA) secrete radiolabeled material into the medium which is mucous glycoproteins based on the following criteria: (a) high molecular weight (excluded from Sepharose CL-4B), (b) emerges unretarded from DEAE-Sephacel under conditions where all proteoglycans bind to the column, (c) incorporates radioactivity from ^3H -glucosamine as N-acetylglucosamine and N-acetylgalactosamine as determined by strong acid hydrolysis and paper chromatography of the component sugars. Cells grown in the absence of RA produce less than 10% of this material compared to RA-treated cells. Cells grown on FAV plates produce undetectable levels of mucous glycoprotein whether or not RA is present in the medium. Ultrastructural analysis of RbTE cells grown under the conditions described above confirms that only cells grown on collagen gel in the presence of RA contain polarized cells with densely staining granules, similar to secretory cell in vivo. Without RA or on FAV, the cells exhibit a layered, squamous morphology, like that of a keratinizing epithelium.

These results indicate that both retinoids and the proper substratum are important for the production of mucous glycoproteins in vitro. These same factors also influence the keratinocyte-type pathway of differentiation. RbTE cells, after plating on FAV dishes, undergo a proliferative period where the cells are rounded, migratory, poorly adhesive and do not form cross-linked envelopes. After reaching the stationary phase of growth, the cells are more adhesive, form flattened colonies and ultimately give rise to cross-linked envelopes. Retinoids not only inhibit the formation of cross-linked envelopes but also reduce adhesion to a level indistinguishable from the undifferentiated proliferative cells. Serum, which enhances squamous cell differentiation, also

increases adhesion. With these preliminary indications that cell shape and adhesion correlate with differentiation, we undertook to identify cellular mediators of adhesion as potential markers of differentiation.

Among proteoglycans, hyaluronic acid (HA) production is reduced upon differentiation to 20% of the that produced by proliferative cells. RA treatment restores most of this reduction. Thus HA production correlates well with differentiation as measured by cross-linked envelope formation and this change precedes terminal differentiation by several days. The correlation with adhesion is less good since untreated and serum-treated cells show similar levels of HA, even though serum-treated cells are much more adhesive. Sulfated proteoglycan (chondroitin sulfate, dermatan sulfate and heparan sulfate) production varies somewhat among the undifferentiated and differentiated conditions, with the most pronounced difference being enhanced sulfation in the serum-treated cells, especially in heparan sulfate thus HS may be involved in the especially tight binding to substratum seen in the presence of serum, but perhaps not in any differentiated function.

With respect to cellular glycolipids, a striking correlation existed between adhesion, differentiation and sulfatide synthesis. Relative to undifferentiated proliferative or RA-treated cells, differentiated cells incorporate 25 to 30 times as much radioactive sulfate into sulfated glycolipids. Further analysis of both gangliosides and surface glycopeptides is planned to complete the survey of glycoconjugates that may be involved adhesion or differentiation.

With regard to differences already observed, we intend to confirm that highly sulfated proteoglycans and sulfatides are intimately involved in the adhesion of RbTE cells by examining "footpad" material left behind when cells are detached from the substratum by EGTA. Putative mediators of adhesion should be enriched in such substratum-attached material. Additionally, enzymes which synthesize and degrade these mediators will be measured in cells grown under various conditions to determine what form of regulation might be important in controlling their turnover.

Proteoglycans or sulfatides may also be involved in cell-cell interactions in which cellular glycoproteins may play a role. Measurement of such proteins will be carried out specifically using antibodies against fibronectin, laminin and collagen and in a general way by measuring the binding of radiolabeled cellular products to immobilized sulfatides or proteoglycans, followed by subsequent elution and gel electrophoresis to identify the bound material. The synthesis of laminin by these cells is a particularly attractive possibility since specific laminin-sulfatide interactions have been described by others.

Finally, having characterized the cell surface molecules involved in cell-cell and cell-substratum interactions when the cells are plated on FAV, we propose to examine the same molecules when the cells are plated on collagen gel, to see again if differences exist between conditions which favor mucin production (+RA) and those which don't (no RA).

PUBLICATIONS

Kim, K. C., Rearick, J. I., Nettesheim, P., and Jetten, A. M.: Biochemical characterization of mucous glycoproteins synthesized and secreted by hamster tracheal epithelial cells in primary culture. J. Biol. Chem. 260: 4021-4027, 1985.

Jetten, A. M., and Smits, H.: Regulation of Differentiation of Tracheal Epithelial Cells by Retinoids. In Sporn, M. and Clark, S. (Ed.), Retinoids, Differentiation, and Disease. London, Pitman, 1985, pp. 61-76.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-25022-02 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Study of the Molecular Mechanisms of Action of Retinoids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. M. Jetten Senior Staff Fellow LPP, NIEHS
Others: J. E. Shirley Biological Lab. Tech. LPP, NIEHS

COOPERATING UNITS (if any)

Environmental Carcinogenesis Group, LPP
Dept. of Biochemistry, Duke Medical School

LAB/BRANCH

Laboratory of Pulmonary Pathobiology

SECTION

Cell Biology Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.5

PROFESSIONAL

0.5

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

We have two lines of evidence that retinoids may affect either the phosphorylation or the synthesis of specific substrates of protein kinases. In the first series of experiments we have shown that retinoids inhibit the induction of ornithine decarboxylase activity by phorbol esters and diacylglycerols. We have provided evidence that this induction occurs via the activation of protein kinase C. Retinoids don't affect the binding of the phorbol esters to its receptor nor does it change the activation of soluble protein kinase C. These results indicate that retinoids act at a step beyond the activation of protein kinase C. Evidence supporting this idea comes from our studies examining the action of retinoids on the reduction of EGF binding by diacylglycerols. This reduction is a result of the direct phosphorylation of the EGF-receptor by protein kinase C. Retinoids are unable to antagonize this action suggesting that also in the whole cell retinoids do not affect protein kinase C activity. In the second series of experiments we examined the action of retinoids on cell proliferation of cells harboring the v-Ha-ras or v-src oncogenes. We have shown that in the cells expressing v-src retinoids inhibit anchorage dependent and independent growth and inhibit ornithine decarboxylase activity whereas in cells expressing v-Ha-ras retinoids have the opposite effect. These results indicate that the same cell line transformed by two different oncogenes respond quite differently to retinoic acid. Retinoids appear not to affect the synthesis of the oncogene products. These experiments indicate that for protein kinase C retinoids act at a step beyond the activation of the kinase. We are examining now whether retinoids interfere with the synthesis or phosphorylation of specific substrates for protein kinases C and pp60^{src}.

PROJECT DESCRIPTION

Retinoids, analogs of vitamin A, induce a variety of biochemical and biological changes in eukaryotic cells. It has been demonstrated in many cell systems that these compounds modulate cellular proliferation and differentiation. The molecular mechanisms of these compounds are not yet fully established. In vivo vitamin A is delivered to cells complexed to a specific binding protein SRBP. There is some evidence that a specific receptor for this complex is present on the surface of target cells. Little is known about what happens after the interaction with such putative receptors. Whether biochemical targets are present in the membrane or whether the action is transmitted further via the mediation of the intracellular binding proteins is not understood. The action of retinoids on cellular proliferation and differentiation predict that these compounds either directly or indirectly affect gene transcription. It has been hypothesized that the intracellular binding proteins CRBP and CRABP mediate the translocation of retinoids to the nucleus where they may modulate gene transcription. Our objectives are to understand (1) what is the role of SRBP in the mediation of the action of retinoids? And what is the identity of the cell surface receptor? (2) Is there a role for metabolites in the mechanism of action of retinoids? (3) Does the cellular retinoid-binding protein complexes interact with specific components in the nucleus? (4) How does vitamin A affect some key biochemical reactions in the cell?

What biochemical reactions are affected directly by retinoids? Ornithine decarboxylase activity and levels of polyamines have shown to regulate cellular proliferation and differentiation and to form a good in vitro and in vivo correlate with tumor promotion. Retinoids have been shown to affect ODC activity and it has been suggested by several investigators that this inhibition may be related to the biological effects of these compounds. We set out to examine (1) at what level retinoids affect ODC activity, (2) whether the action of retinoids on ODC are related to their effect on differentiation and proliferation. To examine the mechanism by which retinoids inhibit ODC we used the induction of ODC by phorbol esters as a model system. We have shown that retinoids inhibit this induction in rat tracheal epithelial cells and a comparison of the structure-function relationship indicates that the receptors for this group of compounds are involved. We have provided evidence that the TPA-receptor is indeed protein kinase C and that its activation is involved in the induction of ODC. We showed that the structure-function correlations for various diacylglycerols and analogs indicate that compounds most active in stimulating protein kinase C and inhibiting ³H-PDBu binding are also the most effective in inducing ODC activity, implicating a role for protein kinase C in this response. Like the action of phorbol esters on ODC, retinoids inhibit the induction of ODC by diacylglycerols. Others have shown that the induction of ODC is related to an increase in the transcription of the ODC gene. Therefore, the inhibition of ODC by retinoids could occur at several levels: inhibition of the activation of protein kinase C, interference with phosphorylation reactions involved in transmitting the signal to the nucleus, inhibition of gene transcription or inhibition of the enzyme itself. Evidence has been accumulated that retinoids do not affect ODC activity at a post-translational level. We have examined whether retinoids

affect the activation of protein kinase C. We have shown that retinoids do not interfere with the binding of phorbol esters to protein kinase C. Moreover, retinoids have no effect on the activation of soluble protein kinase C. These results indicate that retinoids act at a step beyond the activation of protein kinase C. Evidence supporting this idea comes from our studies examining the action of retinoids on the reduction in EGF binding by phorbol esters and diacylglycerols. This reduction is a result of the direct phosphorylation of the EGF-receptor by protein kinase C. Retinoids are unable to antagonize this action of phorbol esters and diacylglycerols suggesting that also in the whole cell retinoids do not affect protein kinase C activity.

Is the response of cells to retinoids dependent on the oncogene(s) expressed? We and others have shown that retinoids inhibit the transformed phenotype of certain cell lines while enhancing or having no effect on the transformed phenotype of other cell lines.

We have hypothesized that this variation in response may be related to different oncogene(s) expressed in these cells (Jetten, A.M., Maturation and Growth Factors, Vol. 3, 346, 1985). To test this hypothesis we examined, in collaboration with Tona Gilmer, the action of retinoids on a Syrian hamster cell line and several transformed neo^R clones obtained after co-transfection of the parental line with pSV2-neo and v-Ha-ras or v-src DNA's.

Treatment of the parental cells or several independently derived cell lines expressing v-src with retinoic acid induced a flatter morphology and inhibited cell growth whereas cell harboring v-Ha-ras had a more spindle-like morphology and were stimulated by retinoic acid. In agreement with the action of cell growth is the effect on ODC activity. Retinoic acid reduces ornithine decarboxylase (ODC) activity in parental cells and cells expressing v-src and inhibited the induction of ODC activity by the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). In contrast retinoic acid enhanced ODC activity in cells expressing v-Ha-ras and acted synergistically with TPA. Both cells expressing v-src or v-Ha-ras were able to grow in soft agar. Retinoic acid inhibited anchorage-independent growth of the cells expressing v-src but enhanced the anchorage-independent growth of cells expressing v-Ha-ras. These results clearly show that the same cell line transformed by two different oncogenes respond quite differently to retinoic acid.

In order to determine at what level retinoids affect oncogene expression the synthesis of the oncogene product in retinoic acid treated and untreated cells was determined. These results showed that retinoic acid does not affect the synthesis of either pp60^{src} or p21, the products of the src and ras oncogenes, respectively. This indicates that retinoids act at a level beyond the synthesis of the oncogene products. In the case of pp60^{src}, which is a protein kinase, retinoids could affect either the synthesis or phosphorylation of a substrate of the pp60^{src} kinase.

We have shown for protein kinase C that retinoids act at a step beyond the synthesis or activation of this kinase and concluded that retinoids might interfere with either the phosphorylation or synthesis of a crucial substrate.

Whether retinoids affect the kinase activity associated with pp60^{src} or act at a level beyond this kinase activity as in the case for protein kinase C has to be determined. We are examining these possibilities by determining the action of retinoids on the phosphorylation of cellular proteins and subsequently by determining the site of phosphorylation and the identity and function of such proteins.

Is there a role for metabolism and/or binding proteins in the mechanism of action of retinoids? We have obtained purified SRBP and are in the process of testing whether this binding protein (1) alters the uptake of retinoids; (2) alters the metabolism of retinoids in the cell using several HPLC-separation techniques to analyze polar metabolites, conversion of retinol to retinoic acid and retinoid-esters; (3) alters or enhances the biological or biochemical response to retinoids. These results should give an insight in the importance of SRBP and metabolism in the action of retinoids. The primary system for these studies will be rabbit tracheal epithelial cells.

To study the role of the cellular binding proteins we will concentrate on the interaction of these proteins with the nucleus and in particular chromatin. Using CRABP and CRBP we will examine the specific interaction of radiolabeled retinoids bound to these binding proteins with isolated nuclei and chromatin. We will determine the site of interaction, whether the retinoid-binding protein complex binds to naked DNA or nucleosomes, whether the sites are sensitive to protease or DNase digestion. A slightly different approach is to incubate whole cells with radiolabeled retinoids and determine whether radiolabeled retinoids are associated with the nucleus. The sites of interactions will then be characterized. The most promising approach is the use of several affinity-labeled retinoids. We have shown that these compounds have biological activity and can be bound covalently to CRABP. These complexes will be used further in studies examining their interaction with chromatin.

PUBLICATIONS

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-25023-02 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Studies on the Mechanism of Neoplastic Development in Airway Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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	D. J. Fitzgerald	Visiting Fellow	LPP, NIEHS
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	C. Walker	Staff Fellow	LPP, NIEHS
	T. E. Gray	Biologist	LPP, NIEHS
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TOTAL MAN-YEARS

4.6

PROFESSIONAL

2.6

OTHER

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of our studies is to elucidate the cellular and biochemical events during the early post-initiation stages of neoplastic transformation. Following carcinogen exposure clonal density tracheal epithelial (RTE) cell cultures revealed 4 different colony types (types I-IV). Only type III + IV were considered to be transformed as judged by colony size, cell density, labelling index and cell size. Even the most advanced transformed colonies had growth fractions of only 30%, and only ~15% of the cells had colony-forming ability; 40% of the cells showed signs of advanced keratinocyte differentiation. Apparently most of the clonal offspring produced by transformed stem cells has limited replicative potential.

Other studies showed that retinoic acid (RA) irreversibly inhibits transformation of RTE cells at concentrations of 10^{-8} M, even when present in the medium for only 1 week. RTE cell transformants are most sensitive to RA during the first 2 weeks of the post-initiation period and become increasingly resistant with the passage of time.

Studies with tumor promoters showed that normal RTE cells are triggered into cell cycle by tumor promoters, that TPA does not enhance transformation frequency and that it is toxic for preneoplastic RTE cells at concentrations of 1 ng/ml. Surviving cells escaping TPA toxicity are temporarily TPA resistant.

Studies to determine the role of oncogenes in neoplastic transformation of RTE cells have shown that the cellular oncogenes myc, H-ras, K-ras, fos, fms, and raf are expressed in several neoplastic RTE cells lines. The level of oncogene expression at preneoplastic stages is being determined.

PROJECT DESCRIPTION

The evidence is substantial that neoplastic transformation in vivo as well as in vitro is a multistage process. Once a carcinogenic agent has altered the target cell population, progression from one stage to another can either occur spontaneously, i.e., without being effected by known agents external to the carcinogen altered cells or it can be driven by so-called promoting factors which enhance and accelerate the conversion process. Many gaps exist in our understanding of this process of progression: the phenotypic characteristics of preneoplastic stages are ill-defined, we do not know whether there is a constant number of preneoplastic stages for a given tumor model nor do we know how many distinct stages can occur. The cellular as well as the molecular mechanisms of progression are poorly understood.

The overall objectives of our studies are (1) to develop a better understanding of the cellular and biochemical events during the early post-initiation stage of neoplastic transformation. Our hypothesis is that neoplastic transformation is a stem cell disease, i.e., the carcinogen altered stem cells respond abnormally to the physiological regulators of cell replication and differentiation. One of our efforts therefore is concerned with defining the growth and differentiation capabilities of carcinogen-altered stem cells. (2) To characterize the response of carcinogen altered stem cells to inducers of proliferation as well as differentiation and to promoting as well as antipromoting agents. (3) To define the molecular changes occurring at different stages of neoplastic transformation. The experimental model which is used in all of our studies is the rat tracheal epithelial transformation system, a clonal transformation system in which several distinct stages are recognized leading to the evolution of neoplastic cell populations.

Differentiation and Self-renewal Potential of Early RTE Cell Transformants

We have previously described the development of transformed colonies in carcinogen exposed RTE cell cultures which can be scored at 5 weeks after exposure. These dark staining colonies (type III + IV) are rapidly growing and are considered to be transformed because most of them can be subcultured, become immortal and after a number of passages, neoplastic. Besides these very conspicuous transformed colonies, smaller, pale staining and slow growing colonies (type I + II) which we consider to be untransformed also occur in cultures exposed to carcinogens. The significance of these latter colonies is unknown. We decided to characterize the various recognizable colony types asking the following questions: (1) Do the cells in the colonies show any evidence of cellular differentiation? (2) How great is the proliferative activity of the cell population in the colonies? (3) What is the size of the stem cell fraction (clonogenic units) in the various colonies? (4) What is the fate of the various colony types with time in culture and are type I and II colonies precursors of type III and IV colonies?

The morphological studies showed very little evidence for cellular differentiation in colonies of type I and II while colonies of type III and IV showed clear signs of keratinocyte differentiation, based on cornified envelope

formation and staining with anti-keratin antibodies; 40-50% of the cells showed evidence of keratinocyte differentiation by ultrastructural criteria. The determination of ^3H -thymidine labeling indices (24 hrs exposure) showed <10% of the cells labeled in type I colonies and 20-30% of the cells labeled in type II to IV colonies. Stem cell analysis, when conducted under the most growth permissive conditions (plating on irradiated feeder cells) showed that type II and III colonies contained 2-4% and type IV colonies about 15% stem cells (colony-forming cells). When plated under highly selective conditions (growth on plastic) the frequency of stem cells surviving on plastic was .06% for the type II and III colonies and 0.6% for the type IV colonies. Type I colonies were not tested because not enough cells could be obtained. The growth and morphological changes were monitored in >90 individual colonies obtained 5 to 9 weeks after carcinogen exposure. Ninety-six percent of the type I colonies either regressed or remained unchanged; 50% of the type II colonies regressed or remained unchanged, the other 50% progressed to type III and IV colonies; >90% of the type III colonies progressed to type IV and 95% of the type IV colonies continued to expand, maintaining the same morphological characteristics.

These studies prompted us to draw the following major conclusions: (1) Transformed RTE cells have differentiation features similar to skin keratinocytes; (2) The growth fraction even in the most advanced transformed colonies is only about 30% which means that transformed stem cells produce approximately 70% nonreplicating offspring; and only 3-15% of the cells in transformed colonies are clonogenic; (3) All of type I and 50% of type II colonies are nontransformed since they don't grow and many even regress; 50% of type II and all of type III and IV colonies are transformed. In future studies we will explore the differentiation features of transformed cells on the biochemical level and examine their sensitivity to inducers of cell differentiation.

Inhibition of Transformation by Retinoids

Retinoids are highly effective inhibitors of carcinogenesis in some in vivo tumor models but are noninhibitory in others. It is neither known by what mechanisms retinoids inhibit tumor development nor why inhibition is affected in some but not in other systems. It is also not known which stages of neoplastic development are inhibited by retinoids. Our interest in retinoids is two-fold: (1) as a possible chemopreventive agent (inhibition of progression and promotion) and (2) as a tool to analyze the abnormal responsiveness to regulators of cell differentiation of carcinogen altered cells at various stages of transformation. Several years ago we found that the addition of large amounts of retinoids to the diet of rats did not inhibit the induction of respiratory tract tumors by polycyclic hydrocarbons. However, these studies revealed that retinoid deficiency increased the susceptibility to lung cancer induction. We decided to readdress the problem of retinoid inhibition of neoplastic transformation using the in vitro RTE cell system. It was found that 10^{-8} M all-trans retinoic acid (RA) reproducibly reduces the transformation frequency by >90% when present during the entire 5 weeks of the transformation assay. We further found that transformation is inhibited significantly (60% inhibition) when RA is added to the cultures as late as the end of the third week of the transformation assay and that 90% inhibition occurs when it is present only during the first

two weeks of the assay. These results indicate that the inhibitory effect is irreversible. Our studies suggested that the transformation process might be particularly sensitive to the inhibitory effects of RA in the early phases of transformation. To examine this possibility further, we isolated cells from type IV transformants of different ages, i.e., 3, 7, 9 and 12 weeks after carcinogen exposure, replated the cells at clonal densities into medium without or with RA (10^{-10} - 10^{-8} M) and measured colony formation 8 days later. There was a 10- to 100-fold reduction in the sensitivity of cells isolated from transformed colonies as a function of age of the transformed colony suggesting that possibly only the earliest stages of transformation are inhibited by retinoic acid. Future studies will be conducted to determine the mechanisms of RA inhibition of transformation and the mechanism of increasing resistance of later stages of transformation.

Effect of Tumor Promoters on Normal and Transformed RTE Cells

One of the more appealing hypotheses of the mechanisms of tumor promotion developed by investigators studying initiation-promotion in mouse skin, is that tumor promoters such as TPA cause terminal differentiation in normal cells while initiated cells are insensitive to this effect of TPA and are instead stimulated to proliferate. We know from previous studies that tracheal epithelium initiated by DMBA can be promoted by TPA. We do not know however what stage of neoplastic transformation is affected (early or late stage) nor do we know whether the mechanism of promotion is similar to that proposed for mouse skin.

The purpose of our studies conducted to date was to determine the effect of TPA on survival, proliferation, and differentiation of normal and immortal pre-neoplastic rat tracheal epithelial cells. Normal RTE cells obtained from 8-week-old rats were plated into medium containing TPA at concentrations ranging from 0.1 to 100 ng/ml. A dose-related increase in colony forming efficiency (CFE) was observed at concentrations above 0.1 ng/ml. At 100 ng/ml the CFE was increased 5-fold above that observed in controls. Similar studies were carried out with immortal, preneoplastic RTE cells derived from carcinogen-exposed primary cultures. Upon exposure to 10 ng/ml of TPA, 9 out of 11 cell lines showed a marked decrease in CFE, ranging from a 70% to a >99% reduction. Two cell lines showed only a minor reduction in CFE at this TPA concentration. Teleocidin B was as potent as TPA in causing inhibition of CFE in preneoplastic RTE cells; the potency of 4-O-methyl TPA was approximately 1/100 that of TPA and phorbol was virtually inactive. Further studies suggested that inhibition of clonal growth of the preneoplastic tracheal cells by TPA was due to cell death occurring as early as 2 hrs after addition of TPA to the cultures. Morphological studies support the interpretation that a large number of cells was severely damaged within 2-4 hrs after TPA exposure. The formation of crosslinked envelopes (CLE), a measure of keratinocyte differentiation, was determined in cultures of preneoplastic RTE cells. TPA caused only minor increases in CLE formation indicating that the cells were not dying as a result of TPA-induced terminal keratinocyte differentiation but rather from TPA toxicity. Thus, preneoplastic RTE cells respond differently to TPA than normal RTE cells.

The role of TPA toxicity in promoting preneoplastic RTE cells to the neoplastic state is being investigated. We want to determine whether the cells surviving TPA toxicity have sustained sublethal damage which could play a role in the evolution of the neoplastic phenotype.

Oncogene Activation During Multi-Stage Neoplastic Progression

The molecular mechanisms involved in neoplastic progression are poorly understood. Evidence is emerging from the work of several laboratories supporting the notion that two or more cooperating oncogenes may be required to convert normal primary cells to the neoplastic phenotype. Neoplastic transformation of RTE cells involves at least three phenotypic changes. The first change, recognized 3-5 weeks after carcinogen exposure of normal primary cells, is the appearance of transformed colonies, the enhanced growth (EG) variants; the second change is the immortalization of EG variants and the third change is the subsequent appearance of neoplastic cell variants which form invasive carcinomas upon inoculation into compatible hosts. Because of the clearly defined progression steps, the RTE cell transformation system is an attractive experimental model to investigate the role of oncogenes in various stages of epithelial cell transformation.

Our long-term objective is to define, in molecular terms, the key events occurring during neoplastic progression of RTE cells. More specifically, we want to identify cellular oncogenes expressed by RTE cells at various stages of neoplastic transformation. It will be important to determine whether the same oncogene or oncogenes are regularly expressed in independently transformed clones and whether this occurs always at the same stage of transformation. We will attempt to identify the mechanism responsible for oncogene expression (i.e., mutation, gene amplification, translocation, increased transcription) and will try to determine whether the expression of any given oncogene is the cause or the result of the phenotypic change at which expression is observed.

We selected 13 oncogenes which have been implicated in pulmonary and/or epithelial carcinogenesis by various studies reported in the literature: myb, erb B, fes, fms, fos, abl, myc (c-myc, N-myc, l-myc), raf, and ras (H-ras, K-ras, N-ras). In collaboration with Dr. Tona Gilmer this panel of oncogenes is used to screen for increased expression of cellular oncogenes in transformed RTE cells by RNA slot blot and Northern analysis. Six transformed clones are being examined at the preneoplastic and neoplastic stage and in tumors derived from inoculations of the transformed clones. Those oncogenes found to be expressed will be investigated further (see above). Elevated expression will be quantitated relative to normal RTE cells by Northern analysis and Southern analysis of DNA will be used to determine whether increased oncogene expression is resulting from gene amplification or rearrangements. Karyotypic analyses will be performed to identify double minutes or homogeneously staining regions. Oncogenes expressed at levels similar to those seen in normal cells will be studied for the presence of mutated forms, if possible (e.g., mutated H-ras, codon 12).

So far four tumor derived RTE cell transformants have been studied. RNA slot blot analysis showed expression of 6 out of 11 tested oncogenes. In several cases (H-ras, fms, and raf) expression is markedly higher than in normal RTE cells. These three oncogenes are now being examined to determine whether they are expressed in a stage specific manner. For that reason the relevant cell lines will be studied at early preneoplastic passages.

PUBLICATIONS

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-25024-02 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Early Pulmonary Lesions Induced by Inhaled Inorganic Particles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

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Others:	L. H. Hill	Chemist	LPP, NIEHS
	V. Roggli	Guest Worker	LPP, NIEHS

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LAB/BRANCH

Laboratory of Pulmonary Pathobiology

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TOTAL MAN-YEARS

3.0

PROFESSIONAL

1.0

OTHER

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interstitial fibrosis caused by inhalation of asbestos and silica affects millions of occupationally and environmentally exposed individuals. We have established animal models to elucidate the basic cellular mechanisms associated with alterations induced by inhalation of asbestos and silica. We have documented the initial deposition sites of the particles and the nature of the earliest lesions consequent to deposition. Now we have quantified by autoradiography and ultrastructural morphometry the cellular alterations which occur at alveolar duct bifurcations (i.e., sites of particle deposition) at varying times after a brief exposure (1 or 5 hrs) to chrysotile asbestos. The autoradiography showed that within 24 hrs after a 5-hr exposure, there was a highly significant increase in the number of terminal bronchiolar epithelial cells, proximal alveolar duct epithelial cells and alveolar duct bifurcation epithelial cells which had incorporated tritiated thymidine (³HTdr) into nuclei. In addition, there were significant increases in the number of interstitial cells in all three anatomic compartments. The significance of this finding was demonstrated by morphometric studies of alveolar duct bifurcations. Here we showed that the volume as well as number of epithelial cells and macrophages were increased by 48 hrs after a 1-hr exposure to chrysotile asbestos. One month after this exposure, the numbers of interstitial macrophages and fibroblasts were significantly increased. Moreover, the volume of the noncellular interstitial collagenous matrix was increased, signaling the presence of early asbestos-induced fibrogenesis. Further studies are ongoing to define the mechanisms through which the asbestos stimulates the fibrotic lung response.

PROJECT DESCRIPTION

Inhalation of toxic inorganic particles such as asbestos and silica causes debilitating interstitial fibrotic lung disease in humans and experimental animals. This has been known for decades, yet there is little information available on the basic cellular and biochemical mechanisms through which the particles injure lung cells and subsequently cause disease. To understand the pathogenesis of the pulmonary lesions, it is essential to establish the events associated with (1) initial particle deposition patterns, (2) uptake and translocation of the particles among various cell types and anatomic compartments of the lung, (3) the physico-chemical changes and distribution of intrapulmonary particles and (4) the initial cell-tissue alterations which signal the development of the disease. The first three of these objectives essentially have been met, at least in part, in earlier studies. The fourth objective is ongoing and is reported here.

The early stages of fibrotic lesions (i.e., asbestosis caused by asbestos inhalation) in humans and experimental animals occur initially in the region of bronchiolar-alveolar junctions. Further progression leads to fibrosis of adjacent pulmonary septa in a fashion that appears to radiate from the vicinity of terminal bronchioles. In the advanced stages of asbestosis, the diffuse fibrosis is observed throughout the parenchyma. Although the restrictive symptomatology of asbestosis may not be clinically evident until decades after exposure has commenced, the onset of fibrotic changes must be a much earlier event. In attempts to define the pathogenesis of the earliest lesions of asbestosis, we have shown that inhaled chrysotile asbestos fibers that reach the gas exchange region of the lung preferentially are deposited at alveolar duct bifurcations in the lungs of rats. These studies demonstrated that fiber deposition is greater on first alveolar duct bifurcations (bifurcations closest to terminal bronchioles) compared to the more distal bifurcations of alveolar ducts. Over 75% of first bifurcations of alveolar ducts exhibited more than ten fibers but fewer than 20% of the second or third bifurcations had as much fiber deposition. In addition, consequent to this deposition pattern, macrophages accumulated on the first bifurcations of alveolar ducts following a 1-hr exposure. Cell injury was shown by finding intracellular interstitial microcalcifications in these bifurcations only one month after the 1-hr asbestos exposure. The size (i.e., cross-sectional tissue area) of these first alveolar duct bifurcations was increased significantly in exposed animals two days after the 1-hr exposure. This increase was partially accounted for by the presence of alveolar macrophages and appeared to involve both the epithelium and the interstitium. Currently, little is known about the development of the early lesions of asbestosis. Various mechanisms, including mechanical irritation by asbestos fibers, autoimmune phenomena and macrophage products have been postulated to be the cause of the fibrotic response. To understand the pathogenesis of asbestosis at the cellular level, it is essential to resolve the nature of the early response to inhaled fibers. The current study defines which specific cells and tissue compartments are involved in the acute response to deposition of chrysotile asbestos on alveolar duct bifurcations and demonstrate whether or not these changes resolve, persist or are progressive. Morphometric and autoradiographic techniques were applied to quantitate changes occurring in alveolar macrophages,

epithelium and interstitium in terminal bronchioles and first alveolar duct bifurcations isolated from the lungs of rats which had been exposed to chrysotile asbestos for 1 hr and then held in room air before sacrifice at 1 day, 2 days or 1 month post-exposure.

To determine whether or not pulmonary parenchymal cells were affected by asbestos deposition, asbestos-exposed and sham-exposed rats were injected IP with 2 μ Ci of tritiated thymidine (3 HTdr)/gm body weight 4 hrs before sacrifice by Nembutal injection. Animals were sacrificed after 24, 33, or 48 hrs recovery from a 5-hr exposure. Terminal bronchioles with adjacent alveolar duct bifurcations were selected from embedded lung tissue. Serial, .5 μ m thick, sections as well thin sections from the selected anatomic units were cut and processed for autoradiography. The numbers of radioactive nuclei for each cell type were counted. Thin sections were used to identify cell types.

Results of our studies on asbestos-exposed rats injected with tritiated thymidine show clearly that five hours of exposure to chrysotile asbestos causes increased numbers of epithelial cells lining terminal bronchioles to incorporate DNA into nuclei. In addition, there are significant increases in the number of 3 HTdr-positive cells at alveolar duct bifurcations. The precise nature of these cell types has not yet been determined, but it appears as though interstitial fibroblasts and epithelial cells are the types most commonly positive as determined by light microscopic autoradiography. No macrophages have been identified with positive nuclei. Interestingly enough, at the first two time periods studied, the epithelial cell populations exhibited most of the positive nuclei; by 48 hrs post-exposure, 3 HTdr-positive nuclei were more prominent in the interstitial cell populations. This correlates with the ultrastructural morphometric data discussed below. Quantitative studies and electron microscopic identification of the first cells which are induced to replicate by asbestos fibers should lead to a more complete understanding of the cellular pathogenesis of asbestos-induced lesions. In addition, knowledge of which cells respond in vivo provides a sound basis for studying these cells in vitro.

Ongoing studies support the hypothesis that brief exposure to asbestos fibers causes injury of epithelial cells lining terminal airways and alveolar duct bifurcations and eventually interstitial fibroblasts. Future studies will clarify the precise cell types affected using light and electron microscopic autoradiography. These studies will be designed to establish whether or not the cell types which exhibit initial incorporation of DNA within the first 24-48 hrs after exposure are the progenitors of the progressive lesions we have measured one month post-exposure.

The nature of the tissue reaction causing enlargement of the first alveolar duct bifurcations was defined using morphometric techniques. Seven-week-old rats were exposed, nose only, for 1 hr to chrysotile asbestos fibers. After the exposure, the animals were kept in air for 2 days or 1 month and then their lungs fixed by vascular perfusion or by intratracheal instillation of 2% glutaraldehyde. The first bifurcation of several alveolar ducts in each animal was isolated from plastic-embedded tissue and thin-sectioned for electron microscopic analysis. Two days after exposure, the volume of epithelium and

interstitium in the duct bifurcations increased by 78% and 28% respectively ($p < 0.05$). The total number and volume of alveolar macrophages on the bifurcations increased about 10 times ($p < 0.05$), while the number and volume of interstitial macrophages increased 3-fold ($p < 0.05$). Statistically significant increases in the numbers of type I (82%) and type II (29%) epithelial cells also occurred. One month after the 1-hr exposure, the volume of epithelium and the number of type I and type II cells were still greater than control values, but these differences no longer achieved statistical significance. The volume of the interstitium, on the other hand, increased 67% ($p < 0.05$) and this was accompanied by a persistently high number of interstitial macrophages, hyperplasia of fibroblasts and accumulation of an increased volume of interstitial matrix.

These results using autoradiography and ultrastructural morphometry demonstrate that a brief exposure to chrysotile asbestos causes a rapid response that involves an influx of macrophages to the first alveolar duct bifurcations and alterations in the alveolar epithelium. These acute structural changes are followed by a progressive response manifested by increased numbers of interstitial fibroblasts and localized interstitial fibrosis.

Future studies will focus upon growth factors and other cell products which induce the pulmonary cells to proliferate and/or produce increased connective tissue components.

PUBLICATIONS

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October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Asbestos Activation of Complement-Dependent Chemotactic Factors for Macrophages

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

3.0

PROFESSIONAL

1.0

OTHER

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We have shown that pulmonary macrophages migrate to the sites where inhaled chrysotile fibers initially are deposited (i.e., surfaces of alveolar duct bifurcations). These macrophages form a major component of an early asbestos-induced interstitial lesion in rats. To establish the basic cellular mechanisms of asbestos-induced lung disease, it is essential to determine the chemical mediators which attract macrophages to these sites of fiber deposition. Chrysotile asbestos fibers, used *in vitro*, activate complement proteins in peripheral blood serum and in lavaged cell-free lung proteins. Fluids lavaged from the lungs of exposed rats contain substantial chemotactic activity for macrophages compared to fluids from sham-exposed animals ($p < .01$). We hypothesize that this chemotactic activity is derived from complement activated by inhaled asbestos on alveolar surfaces. This contention is supported by observing that: Production *in vitro* of chemotactic activity by asbestos in serum or in lung lavageates was blocked by complement inhibitors and that fractionation, by molecular sieve chromatography, of serum proteins and concentrated proteins lavaged from the lungs of asbestos-exposed rats showed that chemotactic activity was detected in the 14-18,000 MW range. This fractionation profile is similar to C5a, the chemotactic product of complement activation. In addition, rats treated with cobra venom factor (CVF) to deplete circulating complement as well as complement-deficient mice demonstrated significantly depressed macrophage accumulation at sites of asbestos deposition. Recently we have learned that the complement-dependent chemotactic factor is activated during a 3-hr exposure to asbestos, peak activity is maintained through 48 hrs post-exposure, but the chemotactic activity is not detectable by 8 days after exposure. Interestingly enough, when CVF-treated rats were exposed to asbestos, their macrophage response returned when circulating complement reached normal levels.

PROJECT DESCRIPTION

Pulmonary macrophages are the cells which form the initial inflammatory response to asbestos inhalation. Our objective is to test the hypothesis that macrophages are attracted to the anatomic sites where inhaled asbestos fibers activate complement-derived chemotactic activity. We have studied the production of complement-dependent chemotactic factors in vivo, in serum and in proteins lavaged from the lungs of normal rats. The findings from these studies then are correlated with morphologic evidence of macrophage accumulation in the lungs of asbestos-exposed animals.

Male rats, 8-10 weeks of age, and congenic strains of C5 sufficient (C5⁺) and deficient (C5⁻) male mice, 4-6 weeks old, were utilized in these studies. Normal rats and rats treated with cobra venom factor, as well as the congenic strains of C5 sufficient and deficient mice were exposed for 3 hrs to chrysotile asbestos in open cages contained within inhalation chambers. Control rats and mice were similarly sham-exposed to room air.

Immediately after, and 48 hrs after a 3-hr exposure to the asbestos aerosol or room air, rats and mice were anesthetized by intraperitoneal injection of sodium pentobarbital. After anesthesia the tracheas were exposed and clamped to prevent lung collapse. The trachea of each animal was cannulated and then perfused by gravity fluid flow of Karnovskys fixative at a pressure of 15 cm H₂O. Thirty minutes later, with the trachea still clamped to prevent collapse, the lungs were removed from the chest cavity and immersed in fresh fixative for 48 hrs. Tissue blocks were processed for scanning electron microscopy (SEM) and dissected to reveal terminal bronchioles and corresponding alveolar ducts.

We have previously demonstrated in rats that pulmonary macrophages migrate to sites of asbestos deposition (i.e., alveolar duct bifurcations) to form a component of an early lesion within 48 hrs after a 1-hr exposure to inhaled chrysotile asbestos. In these studies, we have elucidated the mechanism(s) by which these cells are attracted to sites of fiber deposition. Results of our in vitro studies showed that chrysotile asbestos fibers activated complement-dependent chemotactic activity in serum and lavaged lung fluids. Similarly, in vivo, complement proteins in the lung were activated by inhaled asbestos fibers. This complement activation in vivo and in vitro by asbestos and zymosan generated a low molecular weight chemotactic factor for macrophages. Interestingly enough, the particle-induced in vitro activation of cell-free fluids lavaged from the lungs of unexposed animals was inhibited by prior decomplexation or following incubation with EDTA, a Ca²⁺-Mg²⁺ chelator. Yet, little inhibitory effect was observed following administration of EGTA, a Ca²⁺ specific chelator. Since calcium and magnesium are essential cofactors for the classical and alternative complement pathways, respectively, these findings suggest that chrysotile asbestos fibers activate complement via the alternative pathway in serum and lung fluids to produce complement-dependent chemotactic factors. This is consistent with previous studies on serum-complement activation, wherein chrysotile asbestos was shown to induce the production of C5a by the alternative pathway.

Inhalation studies were carried out to investigate the role of complement, particularly C5, in stimulating the recruitment of pulmonary macrophages to sites of asbestos deposition. Congenic strains of C5-sufficient (C5⁺) and -deficient (C5⁻) mice as well as normal and CVF-treated rats were exposed to chrysotile asbestos. The numbers of macrophages at sites of fiber deposition were significantly depressed in the C5⁻ mice and CVF-treated rats. This result was not due to intrinsic functional impairment of the C5⁻ and CVF-treated macrophages, since these cells demonstrated a normal chemotactic response to zymosan-activated sera. Moreover, the migration of macrophages to sites of fiber deposition in the CVF-asbestos-exposed rats was still depressed 96 hrs after exposure, indicating that this deficit probably could not be ascribed simply to a CVF-induced delay in the time course of the macrophage response. It should be noted that the numbers of accumulated macrophages in the complement-deficient animals were significantly elevated over those observed in the sham-exposed groups. This suggests that a chemotactic factor, other than the complement-dependent one demonstrated here, could be present in exposed animals. This is not surprising inasmuch as it has been demonstrated that asbestos-exposed macrophages secrete chemotactic factors for other macrophages after chronic exposure. Our most recent evidence shows that detection of the complement-dependent chemotactic factor in lavage fluid from our briefly exposed animals precedes the migration of macrophages to alveolar duct bifurcations. Taken together, these results suggest that the initial pulmonary macrophage accumulation at sites of asbestos deposition results primarily, but not solely, from the activation of complement and consequent fiber-induced generation of C5a on alveolar surfaces. Indeed, lavaged proteins from asbestos-exposed animals generated enhanced chemotactic activity that was detected in the molecular weight range of C5a (i.e., 14,000 to 18,000).

Recent studies have shown that chrysotile asbestos fibers activate serum complement to produce factors that stimulate neutrophil chemotaxis. We have shown that pulmonary macrophage chemotaxis is similarly enhanced following in vitro activation of complement in rat serum and in vivo in the lungs of asbestos-exposed animals. Complement proteins are normal constituents of distal lung fluids and are present in biologically active concentrations in lavage fluid recovered from normal animals. Therefore, it seems reasonable to suggest that inhaled asbestos fibers activate complement proteins at sites of fiber deposition. On the other hand, the chemotactic response of pulmonary macrophages to sites of initial particle deposition may not be unique to chrysotile asbestos. A variety of particles, including crocidolite asbestos, cereal grain dust, zymosan, and fly ash, are known to activate serum complement. Therefore, to establish the significance of our findings it will be important to identify the deposition pattern of a variety of inhaled particulates and to determine whether the initial inflammatory response coincides with macrophage migration to sites of particle deposition as demonstrated here after exposure to chrysotile asbestos fibers.

We have not observed increased numbers of neutrophils in the lungs or lavage fluids of rats briefly exposed to inhaled asbestos. Likewise, no significant increase in neutrophils was found in ultrastructural morphometric studies of chronically exposed rats. It seems likely that the deposition pattern and the

cellular nature of the inflammatory response to various agents is dependent in part on the route of administration, i.e., intratracheal instillation of particles in saline versus inhalation of aerosolized particles.

We conclude that the asbestos-activated, complement-derived chemotactic factor is C5a. This may be important in the pathogenesis of lung diseases since it has been shown that complement-associated chemotactic factors stimulate macrophages to secrete lysosomal hydrolases and toxic oxygen radicals. Secretion of these and other products may cause cell injury and facilitate the development of fibrosis, although this contention remains to be proven.

Future studies will be designed to determine which of the many macrophage products induce the epithelial and interstitial alterations defined in the other ongoing studies.

PUBLICATIONS

Warheit, D. B., Hill, L. H., and Brody, A. R.: In vitro effects of crocidolite asbestos and wollastonite on pulmonary macrophages and serum complement. Scan. Elec. Microsc. 2: 919-926, 1984.

Warheit, D. B., George, G., Hill, L. H., Snyderman, R., and Brody, A. R.: Inhaled asbestos activates a complement-dependent chemoattractant for macrophages. Lab. Invest. 52: 505-514, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-25026-02 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interactions of Inorganic Particles with Pulmonary Cell Membranes

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TOTAL MAN-YEARS

3.0

PROFESSIONAL

1.0

OTHER

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inhaled particles such as asbestos and silica are toxic to pulmonary cells. Our hypothesis is that non-specific (i.e., non-receptor mediated) binding and subsequent uptake of positively-charged particles are mediated by negatively-charged cell surface sialic acid groups. In support of this hypothesis, we have shown that chrysotile asbestos causes damage to erythrocyte membranes through binding to terminal sialic acid (SA) residues. The hemolytic events involved (1) binding of the positively-charged chrysotile fibers to negatively-charged SA groups, (2) rapid (within 5 min) distortion of the cells, (3) redistribution of SA groups, and (4) alterations of intracellular Na^+ , K^+ ratios. Negatively-charged crocidolite asbestos bound to and distorted red cells but had no effect on SA groups or ion flux. To establish whether or not similar mechanisms of membrane injury play a role in particle-induced toxicity of pulmonary cells, we have extended our studies to pulmonary macrophages and have shown the following: (1) Wheat germ agglutinin (WGA), a lectin which binds to sialic acid, is distributed evenly across macrophage surfaces. (2) Positively-charged carbonyl iron (Fe) spheres and chrysotile asbestos fibers bind to macrophage membranes at 4° C and the binding is blocked by a dose-dependent pretreatment of the cells with WGA. Other lectins such as Ricin and ConA do not inhibit binding at comparable doses. (3) Fe-spheres normally are readily phagocytized by macrophages at 37° C. In the presence of WGA, over 90% of the phagocytic activity is blocked, but other lectins have no effect. These studies support our hypothesis that charged surface sialic acid groups play a role in particle binding and phagocytosis. In addition, ongoing studies have shown that particles induce the production of arachidonic acid metabolites by pulmonary macrophages. Induction of these potent mediators of inflammation is stimulated through interactions between terminal sialic acid groups and particles bound to the macrophage membranes.

PROJECT DESCRIPTION

Inhaled inorganic particles are phagocytized by a variety of pulmonary cells including macrophages, epithelial and endothelial cells and fibroblasts. Some particles such as asbestos and silica are toxic and induce cell death. The mechanisms through which the particles bind to cell membranes, are phagocytized and ultimately cause cytotoxicity are largely unknown. To address these issues, which are fundamental in understanding the pathogenesis of particle-induced lung disease, we are studying the mechanisms of particle binding to macrophage membranes, phagocytic events, and subsequent production of arachidonic acid (AA) metabolites and oxygen radicals. Work accomplished to date supports the hypothesis that positively-charged particles bind initially to negatively-charged sialic acid groups on the surfaces of macrophage membranes. Whether or not this event is an essential element in subsequent phagocytosis and cell death remains to be determined. Induction of release of AA metabolites *in vitro* is more clear; moreover, this binding stimulates the release of AA metabolites by the macrophages.

Our current hypothesis states that the toxicity of chrysotile asbestos is mediated through its capacity to bind to sialic acid groups on cell membranes. To address this hypothesis, the following objectives are being pursued:

- (1) to determine whether or not positively-charged particles such as iron spheres (Fe) and chrysotile asbestos bind to the SA groups.
- (2) to test the effects of various lectins on rate and efficiency of phagocytosis by macrophages exposed to various particles.
- (3) to characterize the inflammatory mediators released by pulmonary macrophages and study the mechanisms through which particles stimulate the mediator release.
- (4) to establish the distribution of sialic acid (SA) residues on macrophage membranes before, during and after phagocytosis of toxic and non-toxic particulates.

Objectives 1 and 2 largely have been accomplished, were described in last year's Annual Report and are summarized here.

Wheat germ agglutinin (WGA, a lectin which binds to sialic acid) bound to colloidal gold spheres was found to be distributed evenly over macrophage membranes, on both ruffled and smooth cells. Binding of the Au-WGA spheres was inhibited by pretreatment of the cells with WGA alone. Forty-five minutes after addition of positively-charged iron spheres to culture medium, over 70% of the macrophages contained large numbers of particles. This percentage did not increase after 4 hrs of culture. When the iron particles were added to the cultures at 4° C, no measurable phagocytosis took place and over 90% of the cells had particles bound to the cell surfaces. Vigorous washing did not remove spheres bound to macrophage membranes. When the macrophages were pretreated with WGA before addition of iron spheres at 4° C, particle binding was inhibited in a dose-dependent fashion, e.g., .1 mg/ml of WGA inhibited binding by 90%. The same experiments were carried out using chrysotile asbestos as the positively-charged particle, and the results were essentially identical. As further tests for the role of SA in particle binding, the lectin from Limulus

polyphemus also blocked the binding of positively, but not negatively-charged particles. Lectins which bind to other cell surface sugars were used in the experiments described above. Ricin which binds to beta-galactose, ConA which binds to mannose and bovine serum albumin as a non-specific protein all had no measurable effect on particle binding through a wide range of concentrations. The effect of WGA and the other lectins on phagocytosis was studied. WGA and Limulus lectin clearly inhibited phagocytosis of iron spheres. The other lectins had no measurable effect when the numbers of bound and internalized particles were determined over time. These results support our hypothesis that positively-charged particles bind to negatively-charged sialic acid groups on macrophage membranes. This mechanism of initial binding seems essential in the process of non-receptor mediated, so-called "non-specific" phagocytosis. We propose that these events are integral to the toxicity manifested by a variety of inorganic particulates. Future studies will be directed toward addressing the mechanisms of toxicity.

To pursue Objective #3, we have shown that rat alveolar macrophages release cyclooxygenase and lipoxygenase metabolites during phagocytosis of chrysotile asbestos fibers or carbonyl iron beads. This provides an experimental system in which the relationship between phagocytosis and stimulation of AA metabolism can be studied. We now report that contact of iron beads with the plasma membrane of pulmonary macrophages is a stimulus sufficient to trigger the secretion of PGs as well as metabolites of the lipoxygenase pathway of AA-metabolism. We further postulate that this metabolic stimulation is mediated through binding of the particles with terminal sialic acid residues of the plasma membrane. Despite suppression of phagocytosis with cytochalasin D, the full spectrum of AA metabolites, including lipoxygenase products, was seen when the cells came in contact with carbonyl iron beads. This adds substance to the concept that membrane contact alone triggers stimulation of AA metabolism, as demonstrated originally for prostaglandin production by peritoneal macrophages. The quantity of metabolites produced by particle contact alone was significantly less than that induced by normal phagocytosis. This reduction cannot be explained by cytochalasin D pretreatment of the cells. Even though cytochalasin D alone is practically devoid of stimulatory activity, the pretreated cells, when stimulated by calcium ionophore released more metabolite than normal untreated cells. It is possible that calcium ionophore and iron particles may metabolize different pools of arachidonic acid. We suggest that the reduction in AA metabolite release is related to the finding that carbonyl iron beads, when not ingested, appear to cluster and pile on top of the cells.

We speculate that terminal sialic acid residues are the sites of interaction between carbonyl iron beads and the macrophage plasma membrane. To test this idea, we showed that blocking of sialic acid and N-acetyl glucosamine with the lectin wheat germ agglutinin (WGA) prevents binding and phagocytosis of carbonyl iron beads. Blocking of the N-acetyl glucosamine sites alone with succinylated WGA had no such inhibitory effect. Furthermore, binding of the lectin to sialic acid, but not to N-acetyl glucosamine alone (with succinylated WGA), induces a membrane perturbation which stimulates AA metabolism. However, this stimulus is not as potent as the binding of particles to the plasma membrane, and the signal generated by lectin binding is qualitatively different inasmuch as only small

amounts of products from the cyclooxygenase pathway are produced during the first round of stimulation by WGA, whereas LTB₄ production can be stimulated only if a second challenge of the macrophage is carried out with the lectin.

Particle phagocytosis by macrophages can induce the release of cyclooxygenase or lipoxygenase metabolites; however, phagocytosis per se is not always a sufficient stimulus since active phagocytosis of uncoated latex beads produces no AA metabolites. This suggests the need for adequate receptor stimulation prior to phagocytosis to trigger AA metabolism. So far, the search for such receptors which play a role in AA metabolite release by macrophages during phagocytosis has focused mainly on immunological parameters. However, the role of membrane bound terminal sugars in the initiation of "non-specific" particle phagocytosis by mononuclear cells, and more generally in cell activation, is receiving growing attention. Lymphocytic blastogenic stimulation can be induced by membrane sugar-binding lectins such as PHA and Con A and such stimulation can be accompanied by AA and prostaglandin release. On the other hand, macrophage ingestion of zymosan particles, which consistently induces stimulation of AA metabolism, is mediated by the receptor for mannose/N-acetyl glucosamine. We have shown that the initial binding of positively-charged particles to cell surfaces appears to be mediated by negatively-charged sialic acid residues. For example, chrysotile asbestos fibers bind to and cause rapid redistribution of sialic acid on erythrocyte membranes as reported in earlier annual reports. This causes membrane alterations leading to abnormal ion flux and hemolysis. Chrysotile asbestos and carbonyl iron spheres also bind to sialic acid groups on pulmonary macrophage membranes prior to phagocytosis. As discussed here, WGA prevents particle binding and thus diminishes the stimulation of AA metabolism.

We have demonstrated that AA metabolism is stimulated by contact between particles and membrane-bound sialic acid on pulmonary macrophages. This supports the hypothesis that particle binding to terminal sugar residues is sufficient to cause the release of potent inflammatory mediators. Further work will be necessary to determine whether or not particle type is a factor in contact-induced AA metabolism and if these products play a significant role in the pathogenesis of disease.

PUBLICATIONS

Kouzan, S., Brody, A. R., et al.: Production of arachidonic acid metabolites by pulmonary macrophages exposed in vitro to asbestos, carbonyl iron or calcium ionophore. Amer. Rev. Resp. Dis. 131: 624-632, 1985.

Kouzan, S., Gallagher, J., Eling, T., and Brody, A. R.: Particle binding to sialic acid residues on macrophage plasma membranes stimulates arachidonic acid metabolism. Lab. Invest., In Press.

Kliwer, M., Fram, E. K., Brody, A. R., and Young, S. L.: Secretion of surfactant by rat alveolar type II cells: Morphometric analysis and three dimensional reconstruction. Exp. Lung Res., In Press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-25027-02 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Identification and Characterization of Materials Secreted by Pulmonary Clara Cells

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	P. Nettesheim	Chief	LPP, NIEHS
	L. B. Gilmore	Biologist	LPP, NIEHS

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

3.0

2.5

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The functions of the bronchiolar Clara cell are not known although numerous morphological investigations in many species have led to the hypothesis that the cell is secretory. The objectives of this research are to elucidate the secretory nature of the Clara cell, identify and characterize those secretions and determine their extracellular functions. We have developed a model system for the study of Clara cell functions and metabolism using cells isolated from the lungs of rabbits. Clara cells are dispersed from the lungs with pancreatic proteases and purified to over 90% by using, sequentially, a variety of techniques involving centrifugal elutriation, density gradient centrifugation, differential adherence and *in vitro* cultivation. Incubation of the isolated Clara cells with ^{35}S -methionine for 4 hrs results in the radiolabelling of many intracellular proteins some of which were released into the incubation medium. Biosynthesis and secretion of proteins by Clara cells have been compared with those synthesized and secreted by other isolated and purified pulmonary cells including alveolar macrophages, Type II cells, and basal cells. The labeled cellular proteins and proteins released from the cells were identified by using sodium dodecylsulfate-polyacrylamide gel electrophoresis under reducing conditions followed by fluorography and quantified by microdensitometry. Four major newly synthesized proteins with molecular weights of 9 kd, 30 kd, 90 kd and 180 kd were released from the Clara cells. The 9 kd protein accounted for 42% of the released and 10% of the cellular ^{35}S -protein after 4 hrs of incubation. The 9 kd and 180 kd proteins were not synthesized by any of the other cell types. In addition, antisera prepared against Clara cell antigens were used to identify the presence of the 9 kd protein in lavage effluents from the lungs of rabbits. These data demonstrate that Clara cells make and secrete proteins different from those made and secreted by several other major pulmonary cells and that some of those proteins are present in the pulmonary extracellular lining.

PROJECT DESCRIPTION

The pulmonary functions of the nonciliated bronchiolar epithelial cells (Clara cells) are obscure. Recent investigations indicate that the cell is a target for a variety of toxic chemicals although the significance of this sensitivity to toxicants in relation to pulmonary functions in general has not been determined. Numerous morphological investigations in a variety of species have led to the hypothesis that the cell is secretory although the nature and functions of those secretions are not known. We have demonstrated that isolated and purified Clara cells synthesize and secrete several proteins. We wish to determine if any of those proteins secreted under in vitro conditions are also secreted in vivo.

Many cell types exist in the airway and alveolar epithelium of the lungs, or all of which could contribute materials to the pulmonary extracellular lining. Our approach to the study of Clara cell secretions has been to isolate Clara cells in a highly purified form and to identify and compare secretory proteins with those produced by other isolated cell types and those present in the extracellular lining of the lungs.

Our first specific objective was to achieve an overall view of proteins synthesized by isolated Clara cells and to identify proteins released by those cells into the incubation medium. In the presence of ^{35}S -methionine three conspicuous proteins with molecular weights of 9 kd, 36 kd, 90 kd, and 180 kd were detected in the medium by use of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions followed by fluorography. These three proteins remain conspicuous even after incubation of the cells for 18 hours with ^{35}S -methionine. The major protein had a molecular weight of 9 kd and accounted for 42% of the total radiolabel incorporated into proteins in the extracellular fluid after 4 hrs of incubation.

Our second objective was to determine how the protein synthesizing activity of isolated Clara cells differed from that of other pulmonary cells. Type II cells, basal cells, and alveolar macrophages were isolated from the lungs of rabbits, incubated with ^{35}S -methionine, and compared with isolated Clara cells. The purity of the Type II cells, basal cells, alveolar macrophages, and Clara cells was 85%, 95%, 97%, and 85%, respectively. The 9 kd protein synthesized and released by isolated Clara cells did not appear to be synthesized by the other cell types.

Our third objective and that which will receive primary attention in the next year is to identify which of the proteins present in the pulmonary extracellular lining are secreted by Clara cells. Two-dimensional electrophoresis involving non-equilibrium pH-gradient electrophoresis in the first dimension and SDS-PAGE under reducing conditions in the second dimension has revealed that the 9 kd protein synthesized by Clara cells consists of three isoforms. Antiserum prepared in goats against the 9 kd protein indicates that the three isoforms synthesized by Clara cells are also present in lavage effluents from the lungs. Therefore, it appears likely that Clara cells synthesize and secrete these three low molecular weight proteins into the pulmonary extracellular lining. This

antiserum will also be used to identify the cellular and subcellular localization of the 9 kd proteins by immunohistochemical and subcellular fractionation procedures.

PUBLICATIONS

N/A

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES-25028-02 LPP
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Basis for Cellular Changes in Chemical Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
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Others:	M. Koi M. Oshimura L. Annab P. Lamb	Visiting Fellow Expert Biological Lab. Tech. Biological Lab. Tech. LPP, NIEHS LPP, NIEHS LPP, NIEHS LPP, NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pulmonary Pathobiology		
SECTION Environmental Carcinogenesis Group		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS 4.5	PROFESSIONAL 2.5	OTHER 2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Neoplastic development of Syrian hamster embryo (SHE) cells is a multistep process. However, the number of steps and the genes or changes involved are unknown. We have compared the susceptibilities of normal and carcinogen-induced preneoplastic SHE cells to transformation following transfection with plasmids of genomic clones of oncogenic viruses: polyoma virus, Harvey murine sarcoma virus (v-Ha-ras), and MC29 virus (pSVv-myc). Normal SHE cells transfected with polyoma virus DNA formed tumors within 3-4 weeks when injected into nude mice. In contrast, SHE cells treated with v-Ha-ras DNA remained nontumorigenic. SHE cells transfected with v-Ha-ras DNA plus v-myc DNA formed tumors with short latency periods. These results suggest that multiple changes or activated oncogenes are required for the neoplastic transformation. To determine whether activation of ras plus myc was sufficient for tumorigenicity, we performed cytogenetic analyses of tumors formed following transfection of Syrian hamster embryo cells with v-Ha-ras plus v-myc DNAs or polyoma DNA alone. Whereas polyoma-induced, tumor-derived cells were diploid, tumors induced by v-Ha-ras plus v-myc oncogenes were monoclonal and had a nonrandom chromosome change, monosomy of chromosome 15. Thus, an additional change, loss of chromosome 15, is required or advantageous for tumorigenicity induced by v-Ha-ras plus v-myc oncogenes. These results suggest that neoplastic progression of normal, diploid cells requires more than two steps under certain conditions. To determine if normal cellular factors or genes can regulate the phenotypic expression of tumorigenicity and/or oncogenes, cell-cell hybrids between chemically transformed SHE cells and either normal or preneoplastic SHE cells were prepared. Anchorage independence and tumorigenicity were suppressed in hybrids between tumorigenic and normal cells and in hybrids between tumorigenic and most but not all pre-neoplastic cells. This suggests that this suppressive ability may be lost during neoplastic progression and represents one step in this process. </p>		

PROJECT DESCRIPTION

Neoplastic development of Syrian hamster embryo (SHE) cells in culture is a multistep process. Following exposure to chemical carcinogens, normal, diploid SHE cells give rise to altered cells which are nontumorigenic but have an increased propensity to become neoplastic. These intermediate cells, termed preneoplastic cells, can be isolated and cloned and exhibit a number of phenotypic differences from SHE cells, including an abnormal karyotype, altered morphology and an indefinite lifespan or immortality in culture. Cellular genes, homologous to retroviral oncogenes, have been identified and it has been proposed that they play a major role in the neoplastic development of cells in vitro and in vivo. To understand the nature of carcinogen-induced events in the neoplastic progression of SHE cells in culture and the role of oncogenes in this process, and to further characterize normal and preneoplastic cells, we have attempted to achieve the following objectives:

1. To determine the role of expression or mutations of oncogenes in the multistep process of neoplastic development of Syrian hamster embryo cells in culture.
2. To determine the effects of chemical carcinogens on the expression and/or mutation of relevant oncogenes during neoplastic progression.
3. To identify factors and genes which regulate the expression of tumorigenicity and/or oncogenes.

An understanding of the relationship between carcinogen and oncogene-induced neoplastic transformation and the number and nature of discrete stages involved in carcinogen- or oncogene-induced progression to neoplasia require a cell culture system in which both agents induce neoplastic transformation and in which both normal and preneoplastic cells can be identified and isolated. The SHE cell system is a useful model for making this comparison because (a) it is well characterized with respect to chemically induced cell transformation, (b) the progression of SHE cells to neoplasia is well characterized, (c) clonally related normal and preneoplastic SHE cells are available for studying the effects of oncogenes, and (d) both normal and preneoplastic SHE cells are good recipients for exogenous DNA.

We have examined the multistage oncogene-induced neoplastic transformation of normal and preneoplastic SHE cells. Our results show that the v-Ha-ras oncogene may be necessary but not sufficient for the neoplastic transformation of preneoplastic SHE cells and that additional change(s) may be required for the development of neoplastic potential. Similarly, in addition to the cooperative action of the v-Ha-ras and v-myc oncogenes, at least one other change may be necessary for neoplastic transformation of normal SHE cells.

We have compared the susceptibilities of normal and preneoplastic SHE cells to neoplastic transformation following transfection by the calcium phosphate precipitate technique with plasmids of genomic clones of three oncogenic viruses: polyoma virus, Harvey murine sarcoma virus (v-Ha-ras), and MC29 virus (v-myc). Normal SHE cells which are stably nontumorigenic in nude mice are competent to take up and express exogenous DNA as demonstrated by transfection experiments with pSV2-neo DNA and certain viral DNAs. Transfection of the cells with 1 µg of polyoma virus DNA, which contains three oncogenes, and then

injection of 4×10^6 transfected cells into nude mice resulted in progressively growing tumors of hamster origin within 3-4 weeks. In contrast, SHE cells treated with 5 μ g of v-Ha-ras DNA remained nontumorigenic. The v-Ha-ras DNA treated cells grew in soft agar at a low frequency, but were morphologically transformed and did not escape cellular senescence. Treatment of SHE cells with v-Ha-ras DNA and v-myc DNA (1 μ g each) and injection into nude mice resulted in tumors within 3-5 weeks after injection. These results suggest that multiple changes or activated oncogenes are required for the neoplastic transformation of SHE cells.

A preneoplastic cell line, DES-4, isolated after treatment of SHE cells with the human carcinogen diethylstilbestrol, was chosen for comparative studies. These immortalized cells are nontumorigenic and excellent recipients for exogenous DNA. In contrast to SHE cells, DES-4 cells were highly susceptible to neoplastic transformation following transfection with either v-Ha-ras DNA or polyoma DNA. Treatment of DES-4 cells with as little as 10 ng of any of these DNAs resulted in a 100% tumor incidence in nude mice. The cells remained nontumorigenic after treatment with v-myc DNA, carrier DNA alone, or plasmid DNA lacking the v-Ha-ras oncogene.

To further investigate the role of the oncogenes in the neoplastic transformation of DES-4 cells, clones of DES-4 cells cotransfected with pSV2-neo and v-Ha-ras DNAs were isolated by antibiotic resistance and characterized. There was a good correlation between tumorigenicity and expression of v-Ha-ras expression in the clones; however, the clones were highly variable in terms of their latency periods *in vivo* and anchorage-independent growth. Neither of these two parameters correlated with v-Ha-ras RNA expression. Cell lines derived from tumors had short latency periods *in vivo*, were highly anchorage independent, and had high levels of v-Ha-ras expression. These results suggest that v-Ha-ras expression is necessary but not sufficient for the tumorigenicity of DES-4 cells and that additional changes in the cells are required. Thus, our findings indicate that in spite of its oncogenic potential *in vivo*, the oncogene of v-Ha-ras alone is insufficient to cause neoplastic transformation of normal or preneoplastic cells in culture. Two steps are required for preneoplastic DES-4 cells to become tumorigenic suggesting that at least three steps may be required for the neoplastic progression of normal SHE under certain conditions.

To determine if the polyoma virus oncogenes or the v-Ha-ras plus v-myc oncogenes were sufficient for neoplastic transformation of normal SHE cells, we examined the karyotypes of cells from tumors which formed. The cells from all six tumors which formed after transfection with the v-Ha-ras plus v-myc oncogenes had a nonrandom chromosome change, i.e., monosomy of chromosome 15, in addition to other random chromosome changes. Further, these tumors appeared to be monoclonal in origin as determined by the nearly homogenous sex chromosome constitution of the cells examined from each of the tumors (i.e., the tumors consisted of either XX or XY cells). In contrast, three of five polyoma-induced tumors were polyclonal in origin (i.e., contained both XX and XY cells). In addition, the modal karyotypes of the cells in all five polyoma-induced tumors were normal diploid.

Future studies will be directed at determining the cooperation of oncogenes in the neoplastic transformation of SHE cells, examining the expression of cellular oncogenes in the neoplastic progression of the cells following carcinogen exposure and relating carcinogen action to oncogene activation.

To determine if normal cellular factors or genes can regulate the phenotypic expression of tumorigenicity and/or oncogenes, cell-cell hybrids between chemically transformed SHE cells and either normal or preneoplastic SHE cells were prepared by fusing cells with polyethyleneglycol and isolating hybrid cells by selection for biochemical markers. Our results to date indicate that anchorage independence which is a good marker for tumorigenicity of these cells is suppressed in hybrids between tumorigenic and normal cells and in hybrids between tumorigenic and most but not all preneoplastic cells. This suggests that this suppressive ability may be lost during neoplastic progression and represents one step in this process. Further studies to confirm this conclusion and to identify these genes are in progress.

PUBLICATIONS

Thomassen, D. G., Gilmer, T. G., Annab, L. A., and Barrett, J. C.: Evidence for multiple steps in neoplastic transformation of normal and preneoplastic Syrian hamster embryo cells following transfection with Harvey murine sarcoma virus oncogene (v-Ha-ras) oncogene. Cancer Res. 45: 726-732, 1985.

Thomassen, D. G., Gilmer, T. G., Oshimura, M., Annab, L. A., and Barrett, J. C.: Multistage Neoplastic Transformation of Normal and Premeoplastic Syrian Hamster Embryo Cells by Viral Oncogenes. In Barrett, J. C., and Tennant, R. W. (Eds.): Carcinogenesis - A Comprehensive Survey: Mammalian Cell Transformation - Mechanisms of Carcinogenesis and Assays for Carcinogens. New York, Raven Press, 1985, In press.

Oshimura, M., Gilmer, T. M., and Barrett, J. C.: Nonrandom loss of chromosome 15 in Syrian hamster tumors induced by v-Ha-ras plus v-myc oncogenes. Nature. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES-25029-01 LPP
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanisms of Neoplastic Transformation by Viral and Cellular Oncogenes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) PI: Tona Gilmer Senior Staff Fellow LPP, NIEHS		
COOPERATING UNITS (if any) Cell Biology Group, LPP		
LAB/BRANCH Laboratory of Pulmonary Pathobiology		
SECTION Environmental Carcinogenesis Group		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS 0.5	PROFESSIONAL 0.5	OTHER
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The product of the Rous sarcoma virus (RSV) oncogene, <u>v-src</u>, is a phosphoprotein, pp60<u>src</u>, which has tyrosine-specific kinase activity and is responsible for RSV transformation. We have examined the effects of <u>v-src</u> following transfection of normal, diploid and preneoplastic Syrian hamster embryo (SHE) cells with RSV DNA, containing the <u>v-src</u> gene. Normal SHE cells transfected with RSV DNA formed tumors with a low frequency (2 tumors out of 10 sites) and after a long latency period (14 weeks) when treated cells were injected into nude mice. In contrast, three different preneoplastic immortal SHE cell lines were highly susceptible to transformation by the <u>v-src</u> oncogene to the neoplastic phenotype. Tumors formed with a high efficiency and a short latency period (<3 weeks). Neo^R clones isolated after cotransfection of SHE cells with pSV2-neo and RSV DNAs did not show any evidence of <u>v-src</u> gene expression. These results indicate that the <u>v-src</u> oncogene was primarily responsible for neoplastic transformation of SHE cells but additional changes were required. To determine if neoplastic transformation by <u>v-src</u> DNA in normal cells is initially suppressed, neo^R clones isolated after cotransfection of pSV2-neo and RSV DNAs into a immortalized cell line, 10W, were examined. Analyses of these clones indicate that RSV sequences are not expressed initially. The mechanism of <u>v-src</u> transformation was compared to the mechanism of transformation of another viral oncogene, <u>v-Ha-ras</u>. Cell lines containing either the <u>v-src</u> gene or the <u>v-Ha-ras</u> gene were analyzed following treatment with retinoic acid. Anchorage-independent growth of cells expressing <u>v-src</u> was inhibited by retinoic acid, whereas anchorage-independent growth of cells expressing <u>v-Ha-ras</u> was stimulated. These results indicate that retinoic acid may be a useful probe for distinguishing between different mechanisms of transformation by these two viral oncogenes. </p>		

PROJECT DESCRIPTION

The mechanisms of cellular transformation by viral and cellular oncogenes are poorly understood. The product of the Rous sarcoma virus (RSV) oncogene, v-src is a phosphoprotein, pp60^{src}, which has tyrosine-specific kinase activity and is responsible for RSV transformation. The transforming protein of Harvey murine sarcoma virus (HaMSV), v-Ha-ras is designated p21^{ras} and binds GTP tightly. In contrast to studies of neoplastic transformation induced by chemical carcinogens or transfection of cells with the ras oncogene, which indicate a multistep, cellular process, studies with RSV suggest that the oncogene of this virus (v-src) is able to rapidly transform cells to the neoplastic state in a single step. The reasons for these different results may relate to intrinsic differences in the oncogene products or to differences in the methods of entry and integration of the oncogenes, i.e., virus infection vis-a-vis DNA transfection, which may result in different levels or regulation of oncogene expression. To understand the critical events in neoplastic transformation by viral oncogenes, we have performed experiments with the following objectives:

1. To compare and contrast effects and mechanisms of transformation by the v-src oncogene with the v-Ha-ras oncogene.
2. To determine the cellular and viral factors which regulate the expression of the v-src oncogene.

The ability of cloned Rous sarcoma virus (RSV) DNA encoding the v-src oncogene to neoplastically transform normal, diploid Syrian hamster embryo (SHE) cells was examined. Transfection of RSV DNA by the calcium phosphate precipitate method into early passage SHE cells resulted in a low but significant number of tumors when treated cells were injected into nude mice. Tumors formed with a low frequency (2 tumors formed out of 10 sites injected) and only after a long latency period (14 weeks). Cotransfection of SHE cells with v-src plus v-myc did not increase the incidence of tumors. Further studies were performed to determine the basis for the inefficient transformation of the normal SHE cells. Neo^R clones isolated after co-transfection of SHE cells with pSV2-neo and RSV DNAs were neither morphologically altered nor immortal and did not contain detectable levels of the v-src gene product. These results suggest that neoplastic transformation by v-src DNA in the normal cells is initially suppressed. However, cells from a v-src-induced tumor expressed v-src RNA, and antibody to v-src protein precipitated from the tumor cells a 60,000 molecular weight protein which displayed protein kinase activity. Karyotypic analyses confirmed that the tumor was derived from Syrian hamster cells and suggested that it was clonal in nature. These results indicate that the v-src oncogene was primarily responsible for neoplastic transformation of SHE cells.

In contrast to the normal SHE cells, three different preneoplastic immortal SHE cell lines were highly susceptible to transformation by the v-src oncogene to the neoplastic phenotype. Transfection of these cells with RSV DNA resulted in efficient induction of tumor formation with short latency periods (3 to 4 weeks). DES-4 cells, an immortalized clone isolated after treatment of SHE cells with diethylstilbestrol were chosen for further studies of the effects of the src oncogene at the clonal level. A total of seventeen neo^R colonies isolated after cotransfection of cells with pSV2-neo DNA and RSV DNAs (neo^R RSV

DES-4 clones) and thirteen neo^R colonies isolated after transfection with pSV2-neo DNA alone (neo^R DES-4 clones) were tested for their ability to grow in soft agar and were assayed for tumor-forming ability by inoculation into nude mice at 2×10^6 cells per site. None of the control neo^R DES-4 clones grew in soft agar (frequency $< 1 \times 10^{-6}$) or formed tumors in nude mice in 20 weeks. Out of seventeen neo^R RSV DES-4 clones, fifteen expressed pp60^{src}, grew in soft agar with frequencies of $> 10^{-2}$ and were tumorigenic in nude mice with latency periods of < 3 weeks. One tumorigenic clone (421) did not express v-src gene sequences initially was not able to grow in soft agar (frequency $< 1 \times 10^{-6}$). However, upon injection of this clone into nude mice, tumors appeared after a 6-week latency period, and the tumor cells expressed v-src RNA.

Our results show several distinctions between v-src and v-Ha-ras oncogenes. First, we could not detect any ability of v-Ha-ras oncogene alone to induce neoplastic transformation of the SHE cells in contrast to our results with v-src. However, unlike our results with v-src, v-Ha-ras did induce morphological transformation of the SHE cells and the morphological transformants expressed v-Ha-ras DNA. Neither v-src nor v-Ha-ras induced immortality of the SHE cells in culture, although the SHE-src-T cells were immortal.

A second difference between v-src and v-Ha-ras was the ability to cooperate with v-myc to induce neoplastic transformation: cotransfection of SHE cells with v-Ha-ras and v-myc efficiently induced neoplastic transformation, while cotransfection with v-src and v-myc under the same conditions was without effect. This result can not be explained on the basis of the inability of the plasmid DNA used to induce transformation, since this v-src plasmid induced neoplastic transformation of SHE cells and was very efficient at transformation of three different preneoplastic SHE cell lines. A comparison of the results with DES-4 cells illustrates this point and demonstrates a third difference between v-src and v-Ha-ras. The v-src oncogene was able to efficiently transform DES-4 cells to anchorage-independent growth and tumorigenicity in an apparently single step. While transfection of DES-4 cells with v-Ha-ras oncogene resulted in neoplastic transformation, expression of the v-Ha-ras gene was apparently not sufficient to transform the cells in a single step. Some of these differences between v-src and v-Ha-ras may be at the level of RNA transcription. We are currently testing this hypothesis with a recombinant v-src gene flanked by Moloney leukemia virus long terminal repeats.

In conclusion, our studies demonstrate that transfection with the v-src oncogene can induce neoplastic transformation of normal, diploid SHE cells. However, the efficiency of this transformation is less than the efficiency of transformation with viral infection, and the process apparently requires a second cellular event which allows expression of the transfected DNA. Immortalized, pre-neoplastic cells are efficiently transformed by v-src in a single step. The difference between these two populations may be in the ability to regulate the expression of the v-src gene.

Further studies of the regulation of v-src gene expression were performed with a preneoplastic cell line, 10W, isolated after treatment of SHE cells with

asbestos. Neo^R RSV 10W cell clones isolated after cotransfection of pSV2-neo and RSV DNAs into 10W cells showed no expression of v-src RNA. Soft agar-derived and tumor-derived RSV 10W cell lines contained amplified RSV DNA (approximately 10-fold more than parental neo^R RSV 10W cell clones) and expressed v-src RNA and pp60^{src}. The amplified RSV DNA was present in double minute chromosomes. RSV DNA methylation patterns were similar when Hpa II/Msp I and Sma I restriction digests were compared between parental neo^R RSV cell clones and soft agar-derived or tumor-derived RSV cell lines. Experiments to determine whether v-src RNA is degraded or is suppressed by protein regulation of transcription are ongoing. Neo^R HaMSV 10W cell clones isolated after cotransfection of pSV2-neo and HaMSV DNAs expressed v-Ha-ras RNA and p21^{ras}, were able to grow in soft agar with a frequency of $>10^{-2}$ and were tumorigenic in nude mice with 2-week latency periods, indicating degradation of RNA or suppression of transfected DNA is not a general property of these cells.

In collaboration with Dr. Anton Jetten, LPP, we have further examined the different mechanisms of transformation by v-src and v-Ha-ras by treating cells containing either RSV or HaMSV DNA with retinoic acid. Several parameters of transformation, such as ornithine decarboxylase activity, cell morphology, cell proliferation and anchorage independent growth were examined. The expression of these phenotypes was inhibited in neo^R RSV DES-4 cell clones after treatment with retinoic acid and was stimulated in neo^R HaMSV DES-4 or 10W cell clones. Retinoic acid did not affect the synthesis of pp60^{src} or p21^{ras} in these cells as demonstrated by immunoprecipitation of the viral oncogenic proteins from cells with or without retinoic acid treatment. These results indicate that retinoic acid is able to discriminate between the mechanisms of transformation by v-src and v-Ha-ras and may prove to be a useful tool in discerning targets of these oncogene products.

PUBLICATIONS

Gilmer, T. M., Thomassen, D. G., Annab, L. A., Oshimura, M., and Barrett, J. C.: Influence of chemical carcinogens and viral oncogenes on the neoplastic progression of Syrian hamster embryo cells. In Bishop, J. M., Greaves, M., and Rowley, J. D. (Eds.): Genes and Cancer, UCLA Symp. on Mole. and Cell. Biol., New Series, Vol. 17, Alan R. Liss, Inc., New York, pp. 203-212, 1984.

Gilmer, T. M., Annab, L. A., Oshimura, M., and Barrett, J. C.: Neoplastic transformation of normal and carcinogen-induced preneoplastic Syrian hamster embryo cells by the v-src oncogene. Mol. Cell. Biol., In Press, 1985.

LABORATORY OF REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY

LABORATORY OF REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY

Summary Statement

Research in the Laboratory of Reproductive and Developmental Toxicology seeks to understand basic mechanisms underlying normal and abnormal development and reproduction. The studies draw mainly on the research disciplines of endocrinology, reproductive physiology, developmental biology, pharmacology, neurochemistry, and cell biology. The research in development concentrates on craniofacial, genital tract, and germ cell differentiation. Reproductive studies are conducted largely to understand neurotransmitter/neuropeptide regulation and function, germ cell biology, and the functional cell biology of estrogens. The toxicological mechanisms associated with perturbations of processes leading to craniofacial teratogenesis, transplacental hormonal carcinogenesis, neurotoxin-induced neuroendocrine dysfunction, and failure of germ cell function are investigated. While research problems are developed indepth, the diversity of scientific disciplines establishes the Laboratory as a unique resource within the NIH. As a component laboratory of the NIH, scientists are cognizant of the human health implications of their basic research which contributes to public health efforts and clinical investigations.

OVERVIEW OF CURRENT RESEARCH EFFORTS

The Laboratory of Reproductive and Developmental Toxicology is organized in four Sections. The research emphases in the Sections are described below.

Developmental Endocrinology and Pharmacology Section

Research in the Developmental Endocrinology and Pharmacology Section focuses on three major topics: (1) Pharmacology of estrogens, including structure-activity relationships (SAR) and target organ specific metabolism. Metabolic studies are directed towards understanding the modification of hormonal activity of different estrogenic chemicals and the induction of long-term defects in cell differentiation, including neoplasia, associated with metabolism of estrogens. SAR studies strive to elucidate the structural basis of estrogenicity. (2) Cell biology of estrogens, including studies of estrogen regulation of protein secretion and cell proliferation. Research is designed to understand the role of growth factors in the mitogenicity of estrogens. Major efforts evaluate receptor biology, protein synthesis control mechanisms, and hormone responsive gene structure and function. These studies utilize in vitro models for hormone action, including primary cultures of mouse uterine and seminal vesicle epithelial cells. (3) Developmental biology of estrogens, including studies to elucidate the mechanisms for estrogen-induction of differentiation defects in the genital tract, and the expression of these alterations.

Results obtained in the last year include the separation of the two chiral forms of the diethylstilbestrol (DES) metabolite and analog, indenestrol, by HPLC; after configurational determinations, the receptor binding characteristics of the different enantiomers were found to differ. The DES analogs provide uniquely powerful tools for elucidating the important structural considerations involved in the hormonal activity of environmental chemicals. Further in vitro studies on the metabolism of DES demonstrated the formation of reactive intermediates in the primary target organ for DES carcinogenicity, the fetal genital tract.

To understand the cell biology of estrogens, an estrogen-stimulated mouse uterine secretory protein ($M_r \sim 70,000$) was purified, characterized and an antibody raised against it. While hormone and tissue specificity studies continue, research emphasis is directed towards obtaining the gene for this interesting protein. Other studies have shown that prenatal exposure to DES permanently alters the estrogen-stimulated secretory pattern of the mouse uterus. Additional research has detected epidermal growth factor (EGF)-like activity associated with epithelial cells in sections of estrogen-stimulated mouse uteri; furthermore, preproEGF mRNA has been shown in mouse uterus by dot-blot hybridization with the cDNA probe. Serum-free culture conditions for mouse uterine epithelial cells have recently been established; these cells have EGF receptors and respond to the peptide.

Developmental treatment with estrogens induces permanent alterations in the genital tract response to secondary hormonal cues; these altered responses include both growth and differentiation. Altered differentiation is, in some cases, associated with rare forms of neoplasia. For example, male mice exposed in utero to DES express lesions resembling rete testes adenocarcinoma.

Experimental Teratogenesis Section

The Experimental Teratogenesis Section conducts basic research to understand at the morphological, cellular and biochemical levels various aspects of normal and abnormal embryonic development, especially relating to craniofacial development. Retinoids are craniofacial teratogens in the human, and our results using mouse whole embryo culture demonstrate that they exert a direct effect on the embryo. Cranial neural crest cells are extremely important in development of craniofacial tissues; our studies using neural crest-derived facial mesenchyme cells in primary culture indicate that this may be the primary target cell for retinoid-induced craniofacial anomalies. Future studies are aimed at determining the effect that retinoids have on extracellular matrix production by these cells and the role that retinoid receptors play in normal and abnormal facial development.

Growth and differentiation of secondary palatal epithelial cells in culture are dependent upon epidermal growth factor (EGF) and a fibronectin-rich extracellular matrix (ECM) substrate. A serum-free culture system is utilized to examine the hormonal influences on normal and abnormal epithelial development in the palate. A major focus relates to the manner by which EGF and α -transforming growth factor (α -TGF) influences epithelial development. Evidence suggests that α -TGF is an important embryonic growth factor,

and studies are in progress at the molecular level to determine when and in what tissues α -TGF and its receptor are expressed during embryonic development.

Extracellular matrix (ECM) components play a critical role in palatal development especially hyaluronate, Types I, III, IV and V collagen, fibronectin and laminin. Studies are in progress using cryostat sections and cultured cells from the secondary palate to localize these macromolecules under conditions of normal and abnormal development. Monoclonal antibodies are being produced to palatal epithelial and mesenchymal cells and will be utilized as probes for normal and abnormal development in vivo and in culture.

The dioxin, TCDD, is a potent cleft palate inducer in the mouse; results indicate that this is due to a receptor-dependent inhibition of palatal epithelial cell differentiation. On the other hand, glucocorticoids, such as dexamethasone, induce cleft palate by a receptor-dependent inhibition of palatal mesenchymal cell growth. Studies are in progress in vivo as well as in cell and organ culture to ascertain specific biochemical events which are altered by TCDD or glucocorticoids in palatal epithelial or mesenchymal cells which ultimately result in cleft palate formation. Glucocorticoids exert receptor-dependent alterations in phosphatidylinositol (PI) metabolism in cultured human embryonic palatal mesenchymal (HEPM) cells by increasing the degradation of pre-existing PI. This HEPM cell line is being used to determine mechanisms of teratogenicity as well as a teratogen screening assay. Chemically-induced growth inhibition of HEPM cells, along with a liver-derived metabolic activating system, has been developed as a short-term screening assay for potential human teratogens.

Gamete Biology Section

Research in the Gamete Biology Section during the past year has emphasized identification of molecules that are specific to male germ cells or associated somatic cells and the development of systems to study these cells and molecules in vitro. Two of the major limitations in the study of gamete biology are the lack of cell-specific biochemical markers and of adequate in vitro systems. Monoclonal antibodies have been produced to isolated cells and molecules and are being used to localize and characterize cell-specific and stage-specific antigens. These highly selective probes can be used to study the distribution, synthesis, regulation, and roles of molecules of interest in reproductive and developmental processes. Antibodies have been developed against spermatogenic cells, Sertoli cells, Leydig cells and spermatozoa and are being characterized as to specificity and usefulness for future studies.

Antibodies to cell-specific surface and cytoskeletal antigens of mouse spermatogenic cells are available and efforts are continuing to produce antibodies to stage-specific surface antigens. Antibodies are being produced to Sertoli cells to study essential interactions between these cells and germ cells within the seminiferous epithelium and to monitor the isolation of Sertoli cells and germ cells for biochemical and in vitro

studies. Potentially useful antibodies to surface and cytoplasmic components are being identified. Most of the antibodies to rat Leydig cells also react with mouse Leydig cells and some recognize corpus luteal cells. These antibodies will be used to study development of Leydig cells, morphogenesis of the testis, and the influence of germ cells on steroid production by Leydig cells. Antibodies to spermatozoa are being used to examine modification of the sperm surface during epididymal maturation, which occurs as sperm gain the ability to fertilize, and to study the distribution, synthesis and role of sperm-specific molecules. Other antibodies have been produced against purified antigens, including a sulfated glycolipid unique to the germ cell plasma membrane, a putative sulfotransferase involved in synthesis of this glycolipid and associated with it in the membrane, and an inhibitor of the enzyme. These monoclonals have been generated using a novel modification of the in vitro stimulation procedure, which allows the rapid production of antibodies against remarkably small amounts of antigen.

In vitro studies have involved germ cells separated enzymatically from juvenile and adult mouse testes and the isolation of purified populations by unit gravity sedimentation. Cell viability and metabolism are evaluated by measuring ATP production and synthesis of total and individual proteins to determine optimum medium composition, isolation procedures, and culturing conditions for germ cells. Reasonable viability can be maintained for up to 48 hours. Specific antibody probes will now be used to study synthesis of specific molecules, including cytoskeletal proteins in the sperm flagellum, apparently produced post-meiotically in spermatids, and glycoproteins which appear on the surface of germ cells early in meiotic prophase. Future studies will examine the genes responsible for production of germ cell-specific proteins.

Reproductive Neuroendocrinology Section

Recent research has focused on the mechanisms and interrelationships mediating neuroendocrine responses within the hypothalamic-pituitary-gonadal axis. Studies have been directed toward elucidating the cellular mechanisms involved in the peptide-peptide, peptide-monoamine and peptide-monoamine-steroid interactions governing the regulation of this axis in order to obtain valuable information in the area of neural regulation of endocrine, paracrine and/or autocrine functions. Other studies have been directed towards determining the site(s) and mechanism(s) of action of different endogenous secretagogues and neuromodulators which affect hormone secretion at either the hypothalamic, pituitary or gonadal level. In selected models, an in-depth in vitro exploration is carried out to elucidate the role of specific intracellular mediators responsible for the amplification of different transmembrane signals enhancing hormone secretion. These studies are coupled with in vivo paradigms in order to obtain a measure of the relative physiological significance of these observations in key reproductive events.

One major focus of this research is examining the cellular and subcellular mechanisms regulating the release of luteinizing hormone-releasing hormone (LHRH) and other hypothalamic peptides participating in the modulation of pituitary hormone release. Studies are designed to elucidate the role of

monoaminergic neurotransmitters in the release of LHRH from nerve terminals, the nature of the specific aminergic receptors involved in the neuronal activation that precedes LHRH release, the post-receptor event that participate in the peptide-release process, the role of arachidonate metabolites in amplifying the response to key neurotransmitters, and the additional role played by other intracellular messengers such as Ca^{+2} and other putative intracellular messengers derived from the metabolism of membrane phospholipids.

Other studies have demonstrated that the pattern of hormone-release may be more important than the quantity in determining the response of the target tissue(s). The pulsatile pattern of luteinizing (LH) and follicle stimulating hormone (FSH) secretion will be determined in vivo. Further experiments will examine the effects of altering the parameters of pulsatile input signals on gonadotropin and pro-opiomelanocortin-derived peptide release from incubated pituitaries using a computer-controlled perfusion apparatus.

Additional research involves the analysis of the cellular and molecular mechanisms mediating peptide hormone action. Studies using pituitary cell cultures evaluate the precise mechanisms through which peptidergic or aminergic secretagogues enhance or suppress peptide hormone release. Other studies explore the intratesticular effects of LHRH-analogs which are known to adversely affect both the endocrine and the gametogenic functions of the testis. The interaction of these LHRH-analogs with intrinsic peptidergic systems within the testis such as the pro-opiomelanocortin-derived peptides is also being explored.

Another major focus of research involves the identification of cellular and sub-cellular mechanisms involved in the regulation of pituitary hormone secretion, in particular, prolactin (PRL), adrenocorticotropin (ACTH), and β -endorphin (β -end). Stressful stimuli not only dramatically affect reproductive function as well as the secretion of PRL, ACTH, and β -end, but the stress-induced increase in plasma PRL has also been shown to require a postnatal developmental maturation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70010-09 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Study of Normal and Abnormal Embryonic Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: R. M. Pratt Head, Experimental Teratogenesis Section LRDT NIEHS

Others:	G. K. Andrews	Senior Staff Fellow	LRDT NIEHS
	R. P. DiAugustine	Research Chemist	LRDT NIEHS
	R. I. Grove	Staff Fellow	LRDT NIEHS
	K. S. Morgan	NIH Postdoctoral Fellow	LRDT NIEHS
	T. Watanabe	Visiting Fellow	LRDT NIEHS

COOPERATING UNITS (if any)

Department of Pediatrics
University of Washington, Seattle

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Experimental Teratogenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

6.0

PROFESSIONAL

3.0

OTHER

3.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The purpose of this research project is to understand at the morphological, cellular, and biochemical levels various aspects of normal and abnormal embryonic development, especially relating to craniofacial development. Retinoids are craniofacial teratogens in the human and our results using mouse whole embryo culture demonstrate that they exert a direct effect on the embryo. Cell culture studies with neural crest cells indicate that this may be the target cell type for retinoid-induced craniofacial anomalies. Growth and differentiation of secondary palatal epithelial cells in culture are dependent upon epidermal growth factor (EGF) and a fibronectin-rich extracellular matrix (ECM) substrate. Cyclic AMP greatly enhances the EGF effect and transforming growth factor- α (TGF α), but not TGF β , substitutes for EGF with the palatal epithelial cells in culture. These observations reinforce our hypothesis that TGF α is an important embryo-derived growth factor during development. EGF and TGF α enhance the synthesis of important ECM components in the palate including hyaluronic acid, Type V collagen, fibronectin, and laminin. The dioxin TCDD is a potent cleft palate inducer and our results indicate that this is due to a receptor-dependent inhibition of palatal epithelial cell differentiation. Glucocorticoids exert receptor-dependent alterations in phosphatidylinositol (PI) metabolism in cultured palatal mesenchymal cells by increasing the degradation of pre-existing PI. Chemically-induced growth inhibition of an established line of human embryonic palatal mesenchymal cells, along with a liver-derived metabolic activating system, has been developed as a short-term screening assay for potential human teratogens.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

Normal development of the mammalian embryo is accomplished by a series of extremely complex developmental events including cellular migration, proliferation, recognition, interactions, differentiation, and programmed cell death. The embryo is most sensitive during organogenesis to the adverse effects exerted by various chemicals and drugs (teratogens) which result in birth defects. Facial clefting is a serious and frequently observed human congenital malformation. The etiology of facial clefting is complex but appears to involve both genetic and environmental factors. In order to understand how various teratogens interact with embryonic tissues and disrupt development, we are utilizing a multidisciplinary approach based on mouse palatal development as a model system. Studies are conducted using cell, organ, and whole-embryo culture combined with various methodologies including scanning and transmission electron microscopy, immunocytochemical localization, growth and biochemical analysis, and recombinant DNA technology. The major problem of interest to us is the manner by which various embryonic tissues accomplish a number of difficult and sensitive developmental events, the influence that various hormones and growth factors have on these events, and the mechanisms by which selected teratogens interfere with development.

Objectives

(1) Whole Embryo Culture. Using day 8 to 12 mouse embryos in culture, our research efforts are directed towards understanding the mechanisms by which various teratogens interfere with embryonic development. It is well known that glucocorticoids interfere with development of the murine secondary palate on gestational days 12 through 14. However, little is known concerning the sensitivity of the day 8 to 12 embryo to glucocorticoids, such as triamcinolone. Our objectives are to develop culture conditions that support the growth of the day 8 to 12 embryo, including development of the primary palate, and to ascertain whether glucocorticoids interfere with these events.

Recent clinical observations indicate that the administration during gestation of isotretinoin (13-cis-retinoic acid) for treatment of cystic acne results in a high frequency of craniofacial malformations. Our objective is to produce an animal model in whole embryo culture for this "human retinoid syndrome."

(2) Gene Expression During Development. Studies on the ontogeny and expression of specific genes during development are important for understanding mechanisms of normal as well as abnormal development.

Alphafetoprotein (α FP) and metallothionein (MT) are critical and important proteins produced by the developing visceral yolk sac and liver. Using DNA-recombinant techniques, our aim is to determine the ontogeny and hormonal-responsiveness of expression of α FP and MT genes in the yolk sac and liver during embryonic and fetal mouse development.

(3) Normal and Abnormal Development of the Secondary Palate. Our overall major research objective is to understand the mechanisms of normal and abnormal development of the mammalian secondary palate. Glucocorticoids and dioxins (TCDD) are inducers of cleft palate in sensitive strains of mice; our results indicate that receptor-dependent alterations during palatal development are involved in the etiology of cleft palate. Our studies are aimed at testing this hypothesis critically by localizing glucocorticoid receptors within the palate and determining the level of TCDD receptors in the developing palate from responsive strains of mice.

Epithelial cell growth and differentiation in the secondary palate results in three distinct cellular phenotypes. It is our objective to develop a serum-free cell culture system in which to examine the hormone, growth factor and extracellular-matrix substrate requirements and influences on the isolated palatal epithelia.

(4) Inositol-Containing Lipids In Embryonic Cell Culture. We have postulated that the glucocorticoids produce cleft palate by inhibiting proliferation of the palatal mesenchymal cells. One of our research objectives is to understand, at the biochemical level, the basis for this inhibitory effect. Recent work by others has linked changes in the phosphatidylinositol (PI) cycle at the cell surface membrane to a key role in regulation of cellular proliferation. Our studies are aimed at determining whether or not glucocorticoids alter the PI cycle and subsequent cell proliferation in palatal mesenchymal cells in culture.

(5) Short-Term Teratogenesis Assay. At the present time, there is a great deal of interest in many labs throughout the world in developing short-term, inexpensive in vitro assays that could help to predict those chemicals and drugs of greatest potential risk to the human conceptus (1). Our objective is to use the line of human embryonic palatal mesenchymal cells, that we have previously established in culture, to develop a screening assay based on chemically-induced inhibition of cellular proliferation.

Experimental Approach and Scientific Justification; Recent Accomplishments and Significance

(1) Whole Embryo Culture. Most teratogens exert their effects during the major organogenesis phase of embryonic development which occurs from days 6 to 14 of gestation in the mouse. In vivo approaches to understanding the mechanisms of teratogenesis are useful but limited since manipulation of certain variables, such as length and dose of teratogen exposure, is difficult. In our lab, we have developed conditions for the successful

culture of rodent embryos during days 8-12 of gestation, when the embryo is undergoing its most dramatic growth and organ development. With these culture systems, our studies are designed to understand the mechanisms by which various craniofacial teratogens, such as glucocorticoids and retinoids, affect embryonic development in culture.

Our results demonstrate that early somite (day 8) CD-1 mouse embryos cultured in 100% rat serum for 48 hours grow and develop to the equivalent of day 10 embryos in vivo, whereas day 10 embryos cultured for 48 hours grow and develop best in Waymouth's medium containing 50% fetal calf serum. In order to examine craniofacial development in culture after day 12, we have found that dissected embryonic heads can be successfully cultured in the same roller bottle system as day 8 and 10 embryos using Dulbecco's modified MEM containing 10% fetal calf serum. Using the combined techniques of whole embryo and head culture, we have been able to obtain complete development of the primary and secondary palate in culture. Glucocorticoids exert stage specific effects on embryonic development: heart and neural tube malformations are induced between days 8 and 10 in culture; cleft lip is observed between days 10 and 12 in culture.

Recent clinical observations demonstrate that isotretinoin [13-cis retinoic acid (cRA)], used in the U.S. since 1982 for treatment of cystic acne, is a human teratogen causing primarily heart and craniofacial malformations including ear and palatal defects. In order to develop an animal model in culture, day 8 CD-1 mouse embryos are cultured in the presence of various concentrations of cRA. At 2×10^{-6} M cRA, growth retardation is minimal, and approximately one-third of the embryos exhibit very specific defects including a dramatic reduction in the size of the first and second visceral arches and their derivatives, including the maxilla, mandible, and ear. These malformations would be expected to result in defects similar to those observed in the human, and preliminary observations suggest these defects are due to cRA-induced inhibition of cranial neural crest migration. Using day 10 mouse embryos cultured for 48 hrs., we have found that cRA at 2×10^{-5} M produces a high percentage of embryos with limb defects and median cleft lip. Preliminary observations suggest that cRA specifically inhibits the proliferation of the median nasal facial mesenchyme cells after they have completed their major migration.

(2) Gene Expression During Embryonic Development. The ontogeny and tissue specificity of expression of alphafetoprotein (AFP) and metallothionein (MT) in murine embryos has been studied. The MT gene was found to be expressed in high levels in the endoderm of the visceral and parietal yolk sacs.

Since expression of AFP and MT can be influenced by glucocorticoids, we determined the effects of glucocorticoids on mRNA levels in embryonic and fetal tissues. We found that AFP and MT mRNA levels can be dramatically affected in neonatal liver, but apparently not in embryonic or fetal liver or yolk sac, even with teratogenic doses of hormone. This indicates that the glucocorticoids are not responsible for induction or modulation of

expression of MT or AFP genes during embryonic development. Since glucocorticoid action is a receptor mediated event, we determined the ontogeny of glucocorticoid receptors in the developing yolk sac and liver. The visceral yolk sac was found to contain glucocorticoid receptors from day 14 to 18 of gestation, and the fetal liver contained low levels of receptor which increased dramatically after birth. Our results are consistent with the hypothesis that glucocorticoids do not induce MT or AFP gene expression in fetal liver or visceral yolk sac. However, the presence of glucocorticoid receptors in the visceral yolk sac during midgestation implies a role for glucocorticoids in other yolk sac functions.

(3) Normal and Abnormal Development of the Secondary Palate. Formation of the secondary palate involves a number of interesting developmental phenomena including programmed epithelial cell death, epithelial-mesenchymal interactions, morphogenetic movements, and cellular recognition and adhesion. These events occur in the mouse on gestational days 11 to 14 and are accessible to a number of experimental manipulations including cell, organ and whole-embryo culture.

Glucocorticoids are capable of inducing cleft palate in sensitive mouse strains and appear to directly affect the developing palatal shelves through a receptor-dependent process. Immunocytochemical studies, using polyclonal antibodies against the activated rat liver glucocorticoid receptor, have shown that glucocorticoid receptors appear to be localized mainly in the palatal mesenchymal cells; these results suggest that these are the primary target cells for glucocorticoids with respect to cleft palate formation. Cell culture studies using primary mouse palatal mesenchyme and established human embryonic palatal mesenchyme cells (HEPM) demonstrate that glucocorticoids inhibit both mesenchymal cell proliferation and synthesis of certain extracellular matrix components, including specific types of collagen and glycosaminoglycans. Both of these biochemical effects in vivo result in smaller palatal shelves which are unable to make contact and fuse.

We have developed in vitro conditions under which the growth and differentiation of the isolated palatal epithelia can be examined. The day 12 to 13 palatal epithelium is enzymatically removed from the mesenchyme and allowed to attach to an extracellular-matrix substrate rich in type I and III collagen and fibronectin. Growth factor and hormonal requirements have been determined by culturing in a serum-free media. Epidermal growth factor is an absolute requirement for adhesion, growth and differentiation of the epithelial cells, especially the oral cells which seem to be the most responsive cell type. This is due, in part, to the fact that the oral cells have the highest level of EGF receptors, as demonstrated using labeled EGF binding and subsequent autoradiography. This effect of EGF on the oral cells can be greatly enhanced either by adding cyclic AMP or co-culturing on lethally-irradiated 3T3 embryonic fibroblasts. It appears that there is a stem cell population in the oral epithelium which, under the appropriate conditions, can proliferate extensively in culture.

In the presence of EGF, the medial epithelial cells are responsive and do not undergo programmed cell death, whereas the nasal cells are unresponsive to EGF. In addition, EGF appears to exert an important influence on the extracellular matrix components of the secondary palate; organ culture studies have shown that EGF enhances the synthesis and accumulation of fibronectin, type V collagen, and hyaluronic acid. Previous studies in our lab have provided evidence for the production of an embryonic type of EGF starting on days 11-12 of gestation. We have recently shown that adult EGF does not cross the rodent visceral yolk sac or chorioallantoic placenta and, thus, the postulated existence of an embryo-derived EGF.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent cleft palate inducer in sensitive strains of mice. The strain sensitivity is related to the distribution of the Ah locus. The Ah locus determines the induction of several cytochrome P-450 mediated monooxygenase activities and the major gene product of this locus is the cytoplasmic receptor protein. Our results in CD-1 and C57BL/6J mice indicate that TCDD exerts a direct effect on the developing palatal shelves through a receptor-dependent mechanism that results in a lack of medial epithelial cell death. As a result, the palatal shelves, which are not inhibited in their growth, make contact in vivo but fail to fuse due to the TCDD-induced defect in medial epithelial cell differentiation. Following combined in vivo administration, at subthreshold doses, TCDD and glucocorticoids appear to exert synergistic receptor-dependent effects that result in a high incidence of cleft palate.

(4) Alterations In Inositol-Containing Lipids In Embryonic Cell Culture. Recent evidence has linked the receptor-mediated degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) and/or phosphatidylinositol (PI) to the generation of intracellular secondary messengers thought to play a key role in regulation of cell proliferation. Glucocorticoids markedly alter normal PI degradation and synthesis in a cell line derived from human embryonic palatal mesenchyme. This alteration in PI metabolism correlates in both dose response and time course with hormone-induced inhibition of cell proliferation. More recently, we have used inactive hormone analogs to show that the hormone-induced alteration in PI metabolism is receptor mediated. Furthermore, we have shown the alteration in PI metabolism to be independent of extracellular Ca^{++} concentration, intracellular Ca^{++} -Calmodulin complex formation, and intracellular cyclic AMP levels.

The alteration in PI metabolism is exacerbated by lithium which is known to inhibit a key enzyme in the recycling of the inositol moiety into newly synthesized PI. This finding provides additional evidence that the glucocorticoid-induced alteration in PI metabolism is due to an increase in degradation of pre-existing PI. The correlation between glucocorticoid-inhibited cell proliferation and alteration of PI metabolism has been further strengthened by the finding that stimulators of proliferation, which also stimulates the PI cycle (i.e., serum), are inhibited by glucocorticoids. Stimulators of proliferation which do not stimulate PI metabolism (EGF) are not inhibited by glucocorticoids.

(5) Short-Term Teratogenesis Assays. Exposure to adverse environmental factors is presumably important to the etiology of developmental abnormalities in man. Whole animal tests for teratogenesis are extremely expensive, time consuming, and inadequate to test all the chemicals now in existence and those introduced each year which may present a risk for the unborn. In vitro assays would not eliminate animal testing but would serve to indicate which of the thousands of chemicals that have not been tested should have the highest priority for in vivo testing. Our lab has developed a cell culture assay which is useful in predicting animal and human teratogenesis. This assay is based on the sensitivity of a line of human embryonic palatal mesenchyme (HEPM) cells to chemically-induced proliferation inhibition over a four-day period in culture. Inhibition of proliferation of palatal mesenchyme cells by a number of diverse chemicals and drugs is the major mechanism by which cleft palate is induced in the rodent.

HEPM cells established from a human embryonic palate at eight weeks of gestation may be an ideal cell type to represent undifferentiated mesenchymal cells from a number of embryonic tissues which are frequently malformed in the human. These HEPM cells rapidly proliferate in serum-containing or serum-free media and the karyotype appears normal. To date, we have tested over 65 chemicals and drugs in which there is some information on the animal and/or human teratogenicity. We found a good correlation between the dose which causes a 50% inhibition of cellular proliferation (IC_{50}) and the known drug or chemical teratogenicity. Chemicals and drugs which are poor or weak teratogens have either no effect or an IC_{50} of 1 mM or greater, whereas those which are proven teratogens in rodents and/or human have an IC_{50} of 1 nM to 100 μ M.

The capability of an in vitro system to metabolize proteratogens, such as cyclophosphamide, to its teratogenic form (phosphoramidate mustard) is a highly desirable feature. HEPM cells can be directly exposed to arochlor-induced rat liver S-9 plus cofactors and teratogen for only 4 hours during the day after the cells are seeded onto the culture dishes. Under these conditions, we obtain an S-9 dependent dose-responsive inhibition of proliferation with cyclophosphamide (IC_{50} = 5 μ g/ml).

B. FUTURE PLANS

(1) Role of EGF and TGFs in Palatal Development. The embryonic secondary palate appears to be an ideal tissue in which to examine the role of various hormones and growth factors during embryonic development. EGF has interesting and important effects on both palatal epithelial and mesenchymal cells in cell culture. Our previous studies have indicated that in the rodent, maternal EGF does not cross the visceral yolk sac and/or chorio-allantoic placenta and that the embryo begins to synthesize an embryonic form of EGF at day 11 of gestation. We have proposed that this embryonic

EGF is similar to the sarcoma growth factor, now known as the α - and β -transforming growth factors; these factors may serve as autocrine or paracrine regulators of growth and differentiation in various embryonic tissues including the secondary palatal epithelial and mesenchymal cells.

In order to test this hypothesis critically, we have plans to examine the influence of α - and β TGF in vitro on the growth and differentiation of palatal epithelial and mesenchymal cells. In collaboration with Dr. Richard P. DiAugustine (LRDT) and scientists at the Department of Molecular Biology at Genentech, Inc., who have isolated a cDNA for α TGF, we plan to determine temporal and quantitative aspects of α TGF gene expression in various embryonic tissues including the secondary palate. Antibodies to α TGF will also be used in order to determine its localization in the embryo. In addition, we have obtained monoclonal and polyclonal antibodies as well as cDNA probes to the EGF receptor from the human epidermoid carcinoma cell line (A431); we plan to utilize these antibodies and probes to determine the ontogeny and localization of EGF receptors within various tissues.

(2) Hormonal Influences on Palatal Epithelial Differentiation. One of the major recent accomplishments in our lab has been the development of a serum-free hormone-supplemented culture system in which to examine the growth and differentiation of the isolated palatal epithelium and its three distinct cell phenotypes. Isolated palatal epithelia are cultured on an extracellular matrix substrate which is deposited directly in culture by bovine corneal endothelial cells; this ECM is rich in fibronectin and types I and III collagens. We plan to compare the attachment, growth and differentiation of the palatal epithelia on this ECM to other ECMs including the HR-9-ECM which is rich in laminin and type IV collagen, and the ECMs which are produced by various human embryonic palatal mesenchymal cell lines established in our lab. We are characterizing the biochemical and morphological (SEM and TEM) nature of these matrices using polyclonal antibodies to fibronectin, laminin, types I and IV collagen obtained from the lab of Dr. George Martin at NIH, Bethesda. Furthermore, we are using these antibodies to determine the time of appearance and distribution of these important extracellular matrix components in the rodent and human embryonic craniofacial region as well as the isolated epithelial and mesenchymal cells in culture.

Establishment of human embryonic palatal epithelial cell lines to complement the HEPH cell line will be undertaken in the future. We hope to obtain stem-cell lines which can be influenced, by various hormones and growth factors, to undergo differentiation into either the nasal, oral, or medial epithelial phenotype. EGF has an important role in the growth and differentiation of the oral and medial palatal epithelial cells, whereas we have found that the presumptive nasal cells are not responsive to EGF but are responsive to retinoids and cyclic AMP. The latter are presumably important hormones which influence differentiation of the nasal cells in vivo, and we plan to further explore these observations using our epithelial culture system.

(3) Development of Monoclonal Antibodies to Probe Embryonic Development.

Studies are in progress to develop monoclonal antibodies to various ligands on the mouse palatal mesenchyme cells. They are being produced by immunizing mice with palatal mesenchymal cells and the splenocytes will be fused with myeloma cells to produce antibody producing cell lines. We plan to utilize these antibodies as specific probes for normal and abnormal growth and differentiation of the mesenchyme cells in vitro, as well as in vivo. Similar studies are planned for the palatal epithelial cells. Radio-labeling studies in conjunction with one and two-dimensional PAGE and immunoblotting should enable us to identify the biochemical nature of various cell surface determinants. Addition of specific antibodies to cell, organ, or whole embryo culture will enable us to ascertain possible functional roles for these ligands.

(4) Mechanism of Retinoid-Induced Malformations. Our recent success in reproducing the human retinoid syndrome in mouse whole embryo culture will be pursued in the future. We plan to determine the distribution of retinoids and their receptors in day 8 to 12 mouse embryos as well as to understand how retinoids produce specific effects on migration of embryonic neural crest cells and proliferation of medial nasal mesenchyme cells. We have recently been able to obtain complete development of the secondary palate in culture using a combined whole embryo and head culture system, starting on day 10 in the CD-1 mouse. This system will be further developed and utilized to understand the mechanism of action of cleft palate teratogens (TCDD and retinoids) that appear to act on early palatal development.

(5) Phospholipids and Growth Control. Recent evidence in a number of biological systems strongly suggests a role for the phospholipids, including phosphatidylinositol (PI), in transmitting signals across the cell membrane. Our evidence suggests that glucocorticoids may alter palatal mesenchymal cell growth by disrupting the PI cycle. Future studies will be conducted (collaborating with Dr. Robert Grove at Bristol Myers in Evansville, Indiana) using the human embryonic palatal mesenchyme cells. Further investigation is directed toward determination of specific PI metabolizing enzymes that glucocorticoids may alter. In addition, the effect of glucocorticoids on PIP₂ levels are under current investigation. It is possible that glucocorticoids initially alter PIP₂ levels which result in a change in the equilibrium established between PI \rightleftharpoons PIP \rightleftharpoons PIP₂ and ultimately alter PI metabolism. The results of these investigations are expected to yield additional information on the mechanism by which glucocorticoids alter inositol-containing lipid metabolism.

(6) Studies on Human Palatal Epithelial and Mesenchymal Cells. Validation of our short-term teratogen screening assay using HEPM cell proliferation inhibition is continuing in our lab, as well as at two independent labs contracted by the National Toxicology Program. We are also planning to ascertain the effects of all the known human teratogens in the HEPM system, including the important environmental teratogen, methyl mercury. Additional HEPM cell lines have been established in our lab and we plan to

characterize their hormonal and growth factor sensitivity in culture. Scanning and transmission electron microscopy will be used to ascertain the extent to which human secondary palate development, especially epithelial development, is similar to mouse palatal development.

Human embryonic cells are useful in predicting the teratogenic potential of chemicals and drugs and in understanding the biological mechanism by which chemicals or drugs may disrupt early embryonic development and produce birth defects. Information from our studies may lead to better pregnancy counseling and drug avoidance and may reduce the frequency of congenital malformations.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 70060-12 LRDT
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Developmental Biology/Toxicology of Estrogenic Environmental Chemicals		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) PI: J. A. McLachlan Head, Devel. Endo. and Pharm. Section LRDT NIEHS		
Others: R. R. Newbold Biologist LRDT NIEHS K. S. Korach Research Endocrinologist LRDT NIEHS Y. Tomooka Visiting Fellow LRDT NIEHS C. Bunyagidj Visiting Fellow LRDT NIEHS R. P. DiAugustine Research Chemist LRDT NIEHS C. T. Teng Expert LRDT NIEHS		
COOPERATING UNITS (if any) Bowman-Gray School of Medicine University of Würzburg Duke University Medical Center University of Texas Medical Medical Foundation of Buffalo Center at Houston		
LAB/BRANCH Laboratory of Reproductive and Developmental Toxicology		
SECTION Developmental Endocrinology and Pharmacology Section		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.6	2.8	2.8
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided) <p> Studies have continued to determine the molecular forms and cellular targets of <u>estrogenic chemicals</u> and establish the mechanisms by which interactions of <u>estrogens with developing genital tract target cells</u> result in permanently altered <u>differentiation</u>, including <u>dysmorphology</u> and <u>neoplasia</u>. In the period covered by the report, experiments <u>in vitro</u> with the <u>fetal anlage</u> of reproductive tract tissues from mice, the <u>Müllerian duct</u>, have demonstrated that the fetal tract is imprinted by DES at the <u>molecular level</u> and becomes unresponsive to its normal tissue effector, <u>Müllerian Inhibiting Factor</u>. As a consequence, offspring of both sexes retain <u>genital structures</u> associated with the opposite sex (<u>Müllerian retention</u> in males was associated with <u>epididymal cysts</u> and <u>mesonephric retention</u> in females was associated with <u>paraovarian cysts</u>). An <u>embryologically</u> derived lesion in the male offspring of DES treated pregnancies was detected in the <u>rete testis</u>; lesions resembling <u>rete testis carcinoma</u> (a rare lesion in experimental animals or men) was seen in 5 percent of these treated mice. Furthermore, the response of the <u>uterine epithelium</u> to <u>estrogen</u> at puberty was altered by prenatal DES treatment. The alteration was determined at the <u>molecular and morphological levels</u>. Studies on the <u>target-organ metabolism</u> of DES have revealed <u>uterine specific metabolites</u> both <u>in vitro</u> and <u>in vivo</u>. Furthermore, one pathway in the metabolism of DES and other <u>estrogens</u> occurs via a <u>peroxidase</u> (<u>prostaglandin synthetase</u>) and results, in certain cases, in formation of <u>free radicals</u> and <u>quinones</u>. To study <u>estrogen-induced differentiation defects</u> in detail, <u>serum-free cultures</u> of <u>genital tract epithelial cells</u> have been established. </p>		

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

This project is based on the central premise that exposure to estrogenic chemicals at inappropriate times or amounts during genital tract differentiation will permanently alter the developmental program of these tissues, so that they respond atypically to further stimuli or cues.

The development of the mammalian genital tract involves the differentiation and interaction of two primary embryonic rudiments: the paramesonephric (Müllerian) duct which gives rise to the definitive female sexual structures, including the oviduct, uterus, cervix, and cranial vagina; and the mesonephric (Wolffian) duct, which is the precursor to the male genital tract. Differentiation of the male system involves fetal androgens for maintenance of the Wolffian (male) system and Müllerian-inhibiting substance (MIS) for regression of the Müllerian (female) system. It has been assumed that the absence of these factors results in female genital tract development. The role for estrogens in the process of sex differentiation is poorly understood. Furthermore, the interaction of the Müllerian and Wolffian mesonephric systems in the formation of the definitive sexual structures is complex, involving not only cell-cell relationships but also tissue-tissue associations. The fate of the epithelial and mesenchymal components of the primary embryonic rudiments associated with the female or male sexual structures is only now being determined. Alterations induced in the cells of the developing reproductive tract or changes in the interaction of cell or tissue groups may lead to unexpected responses to secondary hormonal cues later in life. One atypical response in the genital tract may be the disruption of normal differentiation or growth regulation and subsequent neoplasia. It is hoped that principles established for the developing genital tract may provide insights into the mechanisms of estrogen-induced alterations in stem cells which lead to abnormal growth and function in mature tissues.

Objectives

Research in this problem area has four aims:

1. To establish the mechanisms by which interactions of estrogens with developing target cells result in persistently altered differentiation, including neoplasia.
2. To study the manifestations and the stimuli required for the appearance of genital tract dysmorphogenesis.

3. To determine the biologically or toxicologically active molecular forms of estrogenic chemicals and ascertain whether there are similarities between active forms of steroidal and stilbene estrogens.
4. To elucidate the critical targets at the cellular and subcellular levels for estrogenic compounds during development.

Experimental Approach and Scientific Justification; Recent Accomplishments and Significance

The experimental approach utilizes a combination of cell culture, organ culture, and whole animal studies. The complex problem of estrogen induction of differentiation defects is unlikely to be solved if studied wholly in vivo or in vitro. Furthermore, the complementary tools of morphology and biochemistry are being used because the alterations involve heterogeneous tissues in both fetal and adult organs.

Diethylstilbestrol (DES) was chosen for study because of its clinical and environmental importance, apparent pharmacologic simplicity, and known fetal bioavailability. An animal model was established in which pregnant outbred (CD-1) mice were treated with DES during the period of genital tract organogenesis (days 9-16 of gestation) and then used to study DES-induced dysmorphogenesis and transplacental carcinogenesis. These studies provided the first clear description of DES-induced vaginal adenocarcinoma in experimental animals. They also provide important insights for humans exposed in utero to DES by demonstrating subfertility in male and female offspring, epididymal cysts and retained testes in male offspring, and oviductal abnormalities in female offspring. In addition, the mouse model has been used effectively to study the clinically important relationship of vaginal adenosis (a relatively common lesion in DES-exposed females) to vaginal adenocarcinoma (a rare lesion in DES-exposed females).

Although the animal model has been useful for suggesting clinically significant effects of DES, its primary use is to study the persistent changes induced by estrogens in the developing genital tract and the mechanisms for their subsequent expression in the adult.

Mechanism(s) for estrogen-induced altered differentiation. Few previous studies have dealt with the biochemical basis of DES-induced dysmorphogenesis. Use of the in vivo DES-exposed mouse model, in combination with organ culture of the developing genital tract, has demonstrated a persistent alteration in the molecular differentiation of the fetal mouse genital tract detectable by two-dimensional (2D) gel electrophoresis. A protein of 70,000 daltons and pI 5.8 (70K/5.8) was greatly diminished or absent in the fluorograms of gels derived from the genital tract of 16-day-old fetal, 1-day-old neonatal, or 17-day-old immature female mice that had been treated in utero with DES; the protein was present in the gels derived from genital tract tissues of control females at the corresponding ages. These results are consistent with the hypothesis that

some of the structural and functional defects associated with prenatal exposure to DES reside in early alterations in the program of genital tract differentiation ("molecular imprinting"). It is also one of the first examples of a long-lasting defect at the molecular level associated with developmental exposure to DES.

Very recent studies have demonstrated that mitosis in Müllerian and Wolffian duct epithelial cells peaked earlier in DES-treated fetuses than in the corresponding control tissues. Protein maps of these tissues, using 2D gel electrophoresis and silver staining, revealed 2 domains on the gel with proteins common to 16-day-old DES-treated fetal uteri (but not 16-day controls) and 4-day-old neonatal-control uteri. This apparent acceleration in development suggests that DES may alter the timing of critical interrelated events involving differentiation and regression of tissues in the genital tract in the mouse fetus.

In fact, the development of the genital tract in both sexes is altered by DES; increases in mitotic indexes and in hyperplasia are seen in the Wolffian as well as Müllerian ducts. Furthermore, the Wolffian duct persists in DES-treated females and the Müllerian duct persists in similarly-treated males. The failure of the Müllerian duct to regress appears to be associated with changes in the reproductive tract tissue itself since control fetal testes, which actively elaborate MIS in organ culture, did not induce the regression of Müllerian ducts obtained from DES-treated fetuses.

Expression of dysmorphology. The long-term defects in the structure and function of the genital tract of mice treated during development with DES take many forms. Both male and female offspring of mice treated prenatally retained part or all of the genital structures associated with the opposite sex (Müllerian retention in males was associated with epididymal cysts and Wolffian/mesonephric retention in females was associated with paraovarian cysts). The possible retention of cells from the inappropriate duct system which are not apparent microscopically within the genital tissues is of greater fundamental interest. Such heterologous remnant cells may contribute to subsequent long-term dysfunction.

Another expression of dysmorphology in adult mice treated prenatally with DES involves the uterine epithelium. It is characteristically squamous rather than columnar. Experiments in which DES-exposed mice were ovariectomized prepubertally and then treated with estrogen have established that squamous metaplasia is an estrogen-dependent process in 100% of DES-treated females, but not in controls. A single injection of estrogen stimulated expression of squamous metaplasia in prepubertally-ovariectomized, DES-treated females which persisted for at least four weeks in the absence of further endogenous or exogenous estrogenic stimulation. Furthermore, expression of squamous metaplasia was induced in explants of DES-treated mouse uteri carried in the kidney capsule of untreated hosts. The estrogen receptor (ER) levels in the metaplastic uteri are less than in control

uteri and are similar to those measured in vaginal tissue. The proteins secreted into the uterine lumen in response to estrogen stimulation also reflects the altered state of the tissue.

In collaboration with Dr. Christina T. Teng, a protein of 70,000 daltons has been purified from the uterine luminal fluid of the estrogen-stimulated immature (21-23 day-old) mouse. This estrogen-induced 70K protein is also found as a labeled protein in the media of uterine preparations incubated with ^{35}S -methionine. An antibody to this protein has been raised in rabbits. The 70K protein is also observed in uterine luminal fluid of DES-treated mice.

Biologically/toxicologically relevant form of estrogen. DES is metabolized via reactive intermediates in many species including mice and humans.

Organ cultures of fetal genital tracts, but not fetal liver or placenta of mice, oxidatively metabolize DES to two major products, 4'-O-methyl-DES and Z,Z-dienestrol (Z,Z-DIES). The O-methylation of DES has been demonstrated only in genital tissue in vitro and more recently in situ in the uterus of adult mice treated intravenously with DES. Metabolism of DES to Z,Z-DIES results in formation of the DES-free radical and then quinone and is mediated by peroxidases. The peroxidase pathway apparently is associated with estrogen target tissue, and peroxidase activity is increased in mouse uterus by administration of estrogens. The peroxidase-mediated metabolism of DES also has been associated with cooxidation of DES during formation of prostaglandin from arachidonic acid.

Further attempts to elucidate the molecular form of DES associated with differentiation defects have been carried out in collaboration with Dr. J. Carl Barrett (Laboratory of Pulmonary Pathobiology, NIEHS). A series of structurally-related DES analogues was used to induce neoplastic transformation of Syrian hamster embryo (SHE) fibroblasts; morphological transformation frequencies were highest for those compounds which were good substrates for peroxidase, rather than those which had the best binding affinity for the ER. In fact, the SHE cells had no measurable ER (nuclear or cytosolic), no increase in rate of growth with estradiol (E_2) or DES (at high or low serum concentrations), and no decrease in growth rates with antiestrogen treatment. Similar pharmacologic principles have been established recently for steroidal estrogens. Catechol estrogens (better substrates for peroxidase or arachidonic acid-dependent cooxidation, but poorer ER binders than their respective parent compounds) were more efficient inducers of neoplastic cell transformation than the corresponding parent compound.

Cellular targets for estrogens. Cell cultures have been derived from the differentiated structures arising from the Mullerian (uterus) and Wolffian (seminal vesicle) ducts. Epithelial cells from the seminal vesicles of 2-4-month-old mice were enzymatically dissociated, enriched by gradient centrifugation, and maintained in collagen gel cultures with defined (serum-free) media. The epithelial origin of the cells was determined morphologically, immunocytochemically and biochemically. Cells formed

three-dimensional colonies with a lumen in collagen gels. Cell number was increased 8-fold within a 8-12 day culture period in a medium supplemented with epidermal growth factor (EGF) (10 ng/ml), insulin (10 µg/ml), transferrin (10 µg/ml), cholera toxin (10 ng/ml) and hydrocortisone (0.1 µg/ml). The cells required EGF and insulin; the growth-promoting effects of these two peptide hormones were optimized by transferrin, cholera toxin and hydrocortisone. Fetal calf serum did not support growth, rather, it suppressed the stimulated growth observed in serum-free media. A time-course study revealed that a lag period preceded rapid growth.

Likewise, epithelial cells separated from the uterus of immature (21-23 day-old) mice by treatment with trypsin in a calcium/magnesium free salt solution and gradient centrifugation have been maintained on collagen gel in a defined media including EGF, insulin, transferrin, and retinoic acid. The cells grew in "sheet-like" structures on the gel. The cells were also sensitive to the concentration of calcium in the medium; optimal cell growth is obtained at calcium concentrations of 0.05 mM. The cells contained EGF receptors (approximately 52,000 sites/cell) and respond to this factor with enhanced proliferation. Collaborative studies with Dr. Richard P. DiAugustine have posed a role for EGF in estrogen-induced uterine epithelial cell proliferation since estrogen-stimulated mice have EGF in uterine tissue and secretion as demonstrated by immunocytochemistry and radioimmunoassay, respectively.

B. FUTURE PLANS

Mechanism(s) for Estrogen-Induced Altered Differentiation

The purpose of these experiments is to more clearly characterize the early response of the fetal genital tract to DES.

1. Attempts will be made to characterize the 70K/5.8 protein which is persistently diminished by prenatal exposure to DES. Intracellular localization will be conducted using established techniques of cell fractionation of in vitro labeled tissues as well as attempts at its localization in the epithelium or mesenchyme of fetal and immature uteri following cell separation. The relationships of this developmental protein to the estrogen-induced 70K secretory protein purified from immature mouse uteri will be studied; western immunoblots of gels from uterine tissues will be carried out with the antibody to the 70K secretory protein.
2. The apparent early maturation of the fetal genital tract will be examined in two studies. The earliest detectable morphological and histochemical responses of the surface epithelium to DES will be determined, starting with 11-12 day fetal mouse genital ridges and followed through gestation and early neonatal life. Also, the pattern of DES metabolism in DES-treated fetal genital tracts will be

compared with that in tissue obtained from neonatal and immature mice. It is predicted that this will provide a further indication of an early acquisition of mature properties.

Expression of Dysmorphology

To further study the stimuli and sites for DES-induced genital tract dysmorphology, experiments to define "cell lineages" and responses in fetal tissues will be conducted.

1. In collaboration with Dr. Teng, we will study the mechanisms of DES-induced genital tract dysmorphogenesis in mice, utilizing products of the adult derivatives of the Wolffian and Müllerian ducts. These experiments will be conducted using radioimmunoassays (RIA) to monitor and quantitate the proteins and immunocytochemistry to localize them at the tissue level. The probe for the Müllerian duct derivative will be the antibody to the estrogen-induced 70K uterine secretory protein; the Wolffian duct probe will be an antibody to an androgen dependent seminal vesicle secretory protein (SVS IV). The development and validation of the uterine 70K protein RIA is currently underway. The RIA will be used to study the ontogeny of hormone induction of the 70K uterine protein. If these proteins are expressed in hormone-stimulated fetal tissues, they can then be used as markers for normal and abnormal genital tract development, including the interaction of the two duct systems. The antibodies may be useful for detecting, by immunocytochemistry, Wolffian-duct derived cells in the adult female genital tract and cells of Müllerian origin in the male tract.
2. Companion studies, in collaboration with Dr. E. M. Eddy, will involve preparing monoclonal antibodies to Müllerian and Wolffian duct tissue from 16-day mouse fetuses. After screening the antibodies on sections of fetal tissues for tissue specificity, attempts will be made to follow the fate of these fetal antigens during the normal and abnormal differentiation of the genital tract. The underlying difficulty in both approaches (as with any cell lineage study) is that the appropriate marker may not be expressed at the time point being investigated.
3. Further studies to determine DES-induced alterations in hormone response of mature structures derived from Müllerian and Wolffian ducts will utilize polyacrylamide gel analysis of secretory proteins in both uterus and seminal vesicle.

Biologically/Toxicologically Relevant Form of Estrogen

The purposes of the estrogen metabolism studies are three-fold: (a) to study the ontogeny of the metabolic capacity of the uterus towards estrogens (since metabolism of these compounds may determine both carcinogenic potential and hormone activity); (b) to determine if there are unique metabolic

capabilities in immature estrogen target tissues which may render them more susceptible to neoplastic transformation; and (c) to use metabolism as a parameter of ontogeny of hormone response in the developing fetal tissues.

1. O-methylation of DES will be studied in organ culture of fetal, neonatal, immature, and adult mouse uteri. The possible estrogen regulation of this enzymatic step will also be determined initially by studying O-methylation of DES in uteri obtained from estrogen-stimulated mice. The formation and subsequent metabolism of catechol estrogens will be studied in organ culture since these estrogen metabolites have been demonstrated to induce neoplastic cell transformation more effectively than their corresponding parent compounds.
2. Structural analogs of DES, as well as selected steroidal estrogens, which differ in ER binding, uterotrophic activity, and as peroxidase substrates have been used to help elucidate the relative contribution of "estrogenicity" and metabolism to cell transformation in vitro. Parallel studies will be done with the same series of compounds to evaluate their relative neoplastic transformation potentials in vivo. For this study, neonatal mice will be used to avoid some of the pharmacokinetic difficulties associated with pre-natal treatment. Preliminary studies have demonstrated uterine adenocarcinoma occurs in 80% of aged mice treated neonatally with DES. Following experiments to establish the uterotrophic ("estrogenic") activity of each of the compounds in neonatal mice, age-dependent tumorigenesis will be determined in the genital tract of females given equivalent estrogenic amounts of the compounds, where possible.
3. Studies will be conducted to separate the epithelial and mesenchymal components of the newborn mouse uterus by established techniques. The tissues will be recombined and, following the appropriate culture period, the recombinants will be explanted to the kidney capsule of ovariectomized female hosts. Recombinants will be made of in utero DES-treated epithelium with naive stroma or naive epithelium with DES-treated stroma, and the relative contributions of each compartment to DES-induced dysmorphology studied.
4. Uterine epithelial cells will be obtained from neonatally DES-treated mice before their first exposure to ovarian estrogen (younger than day 24 of age) and the growth response to calcium, EGF and estrogens compared to uterine epithelial cells derived from control mice. Regulation of 70K protein secretion will also be compared in the two cell populations. Likewise, epithelial cells from the seminal vesicles of DES-treated male mice will be cultured and secretory protein profiles and controls studied. In both cases, special attention will be given to altered differentiation,

function, and proliferation. Attempts will be made to determine, in these cultures, the altered cell type and its relative contribution to the cell population.

Cellular Targets for Estrogens

The purpose of the experiments is to determine critical subcellular targets for estrogens in fetal cells and to identify the earliest estrogen responsive cells in genital tract tissues.

1. Binding of DES and other estrogens to classical and non-classical subcellular targets will be studied. In collaboration with Dr. Kenneth Korach, determination of nuclear ER will be conducted for genital tract tissues from 16-day male and female fetuses. The characterization of these binding species will be determined as differentiation occurs in the uterus and seminal vesicle to study the variation and regulation of ER during genital tract development. A monoclonal antibody to ER obtained from Abbott Labs will be used to determine the appearance of ER in fetal tissues at times of development earlier than 16 days (before this point in development, Wolffian and Müllerian ducts cannot be separated effectively). An aim is to determine the earliest appearance of ER in cells which respond to DES by changes in morphology and increases in mitotic index.

Other studies will consider binding of estrogen to non-classical, non-soluble subcellular targets. The rationale is based on two observations; one related to estrogen-induced growth, the other to estrogen-induced neoplastic transformation. In the first case, estrogen binding to plasma membranes in target and non-target cells may help explain the rapid appearance of EGF observed by Dr. DiAugustine. Additional studies in collaboration with Dr. DiAugustine will attempt to evaluate the role of uterine EGF and its receptor in altered regulation of estrogen-inducible growth in DES-treated mice. In the second case, DES-induced neoplastic transformation has been linked to induction of aneuploidy in cells in culture, and aneuploidy has been induced in vivo by pharmacologic doses of estrogens. Recently, depolymerization of cellular microtubules in vitro after treatment with DES has been demonstrated. These studies suggest that cytoskeletal binding of estrogens is a possible contributor to differentiation defects.

2. Ontogeny of hormone response. The antibody to the estrogen-induced uterine 70K protein will be used to study immunocytochemically the time course for estrogen induction of this secretory response in immature female mice. Of special interest will be the response of the Wolffian duct since the male genital anlagen can be demonstrated to undergo epithelial proliferation with DES administration in utero.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70065-09 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Chemical-Receptor Interactions in Reproduction and Hormonal Toxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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COOPERATING UNITS (if any)

University of Wurzburg Burroughs Wellcome Research Labs
Laboratory of Molecular Biophysics, NIEHS UNC Medical School
Medical Foundation of Buffalo Duke University

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Developmental Endocrinology and Pharmacology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.7

PROFESSIONAL

1.3

OTHER

1.4

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.)

Diethylstilbestrol (DES), a potent synthetic estrogen and reproductive toxicant, has been shown to be extensively metabolized. Some metabolites retain hormonal activity while others are biologically inactive. This assignment of activity is consistent with the receptor binding activity of these compounds. Besides reproductive tract effects, some of these metabolites also elicit neuroendocrine effects by suppressing LH secretion. Two groups of metabolites were found to have poor uterotrophic activity although they bound very well to the receptor. Some of these compounds show a variety of differences compared to the intracellular responses of DES, including lack of receptor synthesis, poor nuclear translocation, excessive retention of the receptor complex in the nucleus and the inability to stimulate certain tissue responses such as DNA synthesis, mitosis, and induction of specific enzymes and proteins.

Estrogen stimulation of reproductive tract tissue involves a mechanism which includes binding to a receptor with subsequent activation and localization in the nucleus. Nuclear translocation follows a bimodal temporal pattern consistent with the stimulation of certain tissue responses such as DNA synthesis or enzyme induction. This pattern was demonstrated by both ligand binding assays and immunoassay with a monoclonal estrogen receptor antibody. Multiple receptor peaks were also present in other estrogen responsive target tissues such as the rat uterus and MCF-7 cell tumors. Only biologically active compounds induce these nuclear receptor events. Both receptor peaks could be demonstrated by direct intraluminal uterine tissue stimulation. The two receptor events appear to be occurring in different uterine cell types with uptake in the stroma first followed by the epithelium. Cell cycle kinetic studies of uterine estrogen stimulation show that the second peak occurs at the beginning of S-phase. A major effect of estrogen on uterine cells was to shorten the cell cycle by contracting the G₁ phase.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

Exposure to environmental agents produces a variety of effects on the reproductive tract, some of which result in infertility and toxicity. In many cases, such effects are direct and produced by agents having estrogenic hormonal activity but with little structural resemblance to the natural ligand. Mechanistically, this hormonal activity is believed to be mediated through an intracellular receptor protein. The receptor demonstrates specific stereochemistry for endogenous compounds but appears to interact less selectively with exogenous chemicals. In order to better understand such differences, studies are needed to determine a structural and chemical basis for the stimulation of estrogenic responses and the involvement of the estrogen receptor in this process. Earlier studies suggested that estrogen hormone action involved a two-step mechanism, in which the ligand was bound by a cytoplasmic form of the estrogen receptor that became activated to a form which translocated into the nucleus where it would interact with the genome. More recent observations using hormone responsive cell cultures have refined the earlier model and suggested that the estrogen receptor may be localized within the nucleus in situ. In either model, nuclear occupancy has been considered paramount for stimulation of tissue responses. Some of the primary responses in the uterus are increased gene transcription and hyperplasia. Past studies have suggested that those responses appear to be stimulated in concert but temporally at different times. It is not understood if the concentration of nuclear receptor at certain times is critical to eliciting the responses or whether a minor population of receptor complex is required at specific gene sites throughout stimulation. Studies are needed to test these different possibilities by evaluating the temporal pattern and biochemical characteristics of various forms of the estrogen receptor during tissue stimulation.

Objectives

- (1) Investigate the structural basis of ligand interactions with the estrogen receptor and develop an understanding of nuclear estrogen receptor interactions and their role in stimulating estrogenic responses.
- (2) Analyze the biochemical properties of the estrogen receptor to determine what processes are involved in its activation.
- (3) Investigate the expression of uterine estrogen responses and evaluate their involvement in uterine stimulation.

Experimental Approach, Scientific Justification, Recent Accomplishments, and Significance

Structure-activity studies using a variety of DES metabolites and analogs

as well as a series of polychlorinated biphenyl (PCB) compounds are being performed to assess the estrogen receptor binding activity, in addition to their ability to localize the receptor in the target cell nucleus and induce hormonal responses. These studies will be performed using a combination in vivo and in vitro approach with uterine tissue and estrogen responsive cells in culture. The DES compounds are an interesting series since they vary structurally and chemically around a common chemical nucleus of DES and exhibit small variations in structure which could result in significant differences in receptor binding affinity and hormonal activity. By using this comparative approach, questions concerning the relationship of ligand binding to the activation state and the genomic interaction of the receptor complex can be answered. If differences are observed, then it should be possible to determine if these different ligand receptor complexes induce hormonal responses differentially or in concert and how this may be related to uterine growth.

DES is a potent synthetic estrogen which also undergoes extensive oxidative metabolism. In order to determine whether the metabolism of DES resulted in hormonally active or inactive metabolites, certain DES metabolites and analogs were tested for estrogenic activity using both an in vivo uterine bioassay and an in vitro receptor binding assay. Results of those studies showed that most of the compounds gave a good correlation between the biochemical and bioassay data. Metabolism involving the stilbene double bond resulted in a progressive decrease in both receptor binding affinity and biological activity. Monocatechol DES and dicatechol DES exhibited reasonable receptor affinity and biological activity, while their respective methylated forms were weak. Certain metabolites, e.g., Z,Z-dienestrol or 1-hydroxy dienestrol, showed weak receptor interactions and poor estrogenicity. This indicates that the metabolism of DES along certain pathways produces inactive metabolites while other pathways do not result in complete hormonal inactivation as seen with estradiol.

Exceptions to the agreement between receptor binding and biological activity were seen with two groups of DES metabolites, indanyl DES isomers and the ψ -DES isomers, which show receptor binding affinities in the range of DES but are 20-200 times less biologically active. Compounds in these groups have subtle structural differences which differ only by positions of a double bond or orientation of a methyl group. Pseudo-DES is an isomeric form of DES in which the 3,4 stilbene double bond is repositioned in one of the side chains. The molecule can exist in either a Z or E form. Receptor interactions of these two isomeric forms showed significant differences with the Z- ψ DES isomer exhibiting a 20-times greater affinity than the E- ψ isomer. This difference was also reflected in weak nuclear receptor translocation, uterine growth, and DNA synthesis stimulation. In order to establish if the receptor binding and biological activity could be explained by structural differences in the two isomers, X-ray crystallographic studies were performed. Comparison of their molecular structures showed a similar crystal structure conformation for both isomers with the rings oriented at right angles to one another compared to the extended planar ring structure of DES. Mathematical transformation programs were used to determine if an extended conformation could be achieved. The computation

demonstrated that only the more active Z- ϕ isomer could demonstrate an extended conformation similar to DES. The use of these ϕ -DES isomers has suggested that a compound does not have to be totally planar in order to have appreciable binding affinity to the receptor. However, it appears that the extended planar structure may be necessary in order to invoke tissue responses.

Indenestrol A (IA) is a DES metabolite which has two other isomeric forms: indenestrol B (IB) where the double bond is shifted and indanestrol in which the double bond is saturated. Saturation of the double bond in indanestrol results in a decreased binding affinity of approximately 100 times that of either unsaturated isomer. In addition, this compound gave very weak biological activity compared to either DES or its unsaturated isomers. However, the indenestrol isomers showed differences in hormone responses compared to DES. IA gave a small increase in DNA synthesis while IB showed an increase which paralleled DES but with a higher dose requirement. Both isomers were effective in stimulating progesterone receptor synthesis; while IA stimulated synthesis of a uterine enzyme end product, glucose 6 phosphate dehydrogenase, IB showed little or no activity. Nuclear receptor studies illustrated that at a comparable dose, IA only translocated 60% of the receptor as IB or DES which were similar. These differences observed from the response data suggest that uterine tissue responses are not stimulated in concert. Secondly, progesterone receptor induction can be dissociated from DNA synthesis which is in opposition to suggested reports from in vitro cell data.

Besides investigating DES compounds, a series of polychlorinated biphenyl (PCB) compounds with different chlorinated substitutions have been assayed for receptor binding and uterotrophic stimulation. PCB's have been shown to cause reproductive toxicity which may be due to inherent hormonal activity, but the mechanism is not well understood, mainly because these chemicals exist as a complex mixture and testing of the hormonal activity of individual compounds has not been done. Receptor binding studies indicated that certain isomers such as 2,4,6 trichloro 4'-hydroxy biphenyl have an activity similar to estradiol, while other isomers with different chlorine substitutions have poor activity. These data suggest that chlorinated biphenyls may interact with the estrogen receptor and this activity is influenced by positioning of the chlorine substitutions in specific spacial orientations relative to estradiol.

Estrogen hormone stimulation has been postulated to be governed by a sustained input mechanism involving nuclear estrogen receptor. However, studies using the DES compounds suggest that elevated nuclear receptor levels are not reflective of hormonal activity (e.g., ϕ -DES), we investigated whether the later second growth response in the uterus may be governed by an additional increase in nuclear receptor. Earlier studies in the mouse uterus demonstrated two peaks of nuclear estrogen receptor after exposure to estradiol. The first was at 30-60 min, and the second was at approximately 7-8 h. Both these nuclear peaks were preceded by decreases in cytosolic receptor levels. Nuclear receptor characterized at both times showed a single high affinity binding component and identical

were further characterized by using a monoclonal estrogen receptor antibody. Results from those studies showed an identical bimodal pattern similar to what was observed with the ligand binding assays. The relationship of this second receptor event to tissue responsiveness was shown by the fact that weak estrogens such as estriol lacked the ability to elicit this second nuclear peak. Only a single high dose of estriol which was uterotrophic also produced the second peak. Injection of a potent uterotrophic estriol derivative (e.g., Nylestriol) with slow release properties showed that the two nuclear receptor peaks remained temporally constant to each other with translocations at 7 h and 16 h, respectively.

The possibility of enterohepatic pharmacokinetics of the estrogenic compounds contributing to the multiple receptor peaks was investigated by performing these uterine temporal pattern distribution studies in germ-free mice which possess no bacterial flora and, consequently, no enterohepatic recirculation. Experiments with the germ-free mice showed the same multiple temporal pattern as non germ-free mice suggesting that these events were not due to a pharmacokinetic anomaly. To evaluate whether systemic factors were involved in producing the multiple receptor peaks, uterine treatment was performed using intraluminal hormone injections. Dose response studies produced a maximal DNA synthesis effect compared to the contralateral saline injected control horn. Nuclear receptor levels under the same stimulation showed a pattern of two peaks at 2-3 h and 9 h. These findings would argue against the possibility that systemic factors were influencing the appearance of these peaks.

Uterine DNA synthesis patterns in the adult ovariectomized mouse show a temporal bimodal pattern with the major peak in activity at 16 h with a second at 24 h. This is earlier than the activity seen in the rat uterus where the first peak is at 24 h. Another approach was used to investigate whether the second peak was associated with uterine DNA synthesis. Low doses of estriol given in two dose combinations at different times showed synthesis response comparable to a single 0 h injection of estradiol. Our newest results have also indicated that the second peak is not unique to the mouse uterus since studies in ovariectomized rats show two nuclear receptor peaks at 0 and 13-14 h. These findings are consistent with the later timing of DNA synthesis in the rat uterus and a requirement for a period of secondary stimulation. In order to evaluate the tissue responsiveness to estrogens in more detail and determine whether the two peaks of DNA synthesis were occurring in different uterine cell types, we investigated the DNA synthesis and mitogenic activity in these uterine cells by thymidine autoradiography. Adult ovariectomized animals show estrogen response, DNA synthesis and mitosis in epithelial cells but not the stroma. Sexually immature animals showed a different pattern where DNA synthesis and mitosis were observed in both epithelium and stroma. By using different age animals, we could demonstrate that stromal mitogenesis is lost between day 28-35 of development and coincides with sexual maturity. Animals which were ovariectomized on day 16 did not show the development of stromal responsiveness but rather gave an adult pattern. These experiments indicate that this tissue responsiveness may be influenced by the ovary.

Altered tissue responsiveness can be induced by estrogen exposure. Mice treated developmentally with DES were used since the uteri are functionally compromised in treated mice. To understand why uteri in some animals were not hormonally responsive, the concentration of estrogen receptors in those animals was examined. Results have shown that animals in the non-responsive group have significantly lower levels of estrogen receptor. These studies suggest that at 1 month of age there is no pattern of receptor differences. By 2-4 months, differences in receptor levels between control and treated groups are noticeable; and by 6 months, significantly lower levels are seen in the DES group. Nuclear receptor assays in these same tissues were low and showed no appreciable differences, indicating that the cytosol levels were not due to differential accumulation. Biochemical characterization of the receptor in the DES group showed similar binding affinities, sedimentation values, and nuclear translocation capacity as controls. Endocrine manipulation using ovariectomy and adrenalectomy can result in an experimental animal with similarly lower uterine receptor levels as the DES animal. However, hormone responsiveness measured as stimulation of uterine weight, progesterone receptor, and DNA synthesis in these animals is not compromised as it is in DES-exposed animals.

Protein synthesis increases dramatically in the uterus after estrogen stimulation, and identification of estrogen responsive proteins are being sought. However, it is not known whether any of these proteins are unique to one cell type. This is particularly interesting since only the epithelial cell shows estrogen responsiveness with DNA synthesis and mitosis. This problem was approached by protein labeling experiments using [^{35}S] methionine and two dimensional gel electrophoresis which have illustrated several proteins (32,000 - 54,000 mw range) in uterine tissue from estrogenized animals. Non-enzymatic separation of the three uterine tissue compartments have indicated that some of these proteins are unique to one cell type. Preliminary results of computer analysis of these gels show that the epithelial and stromal tissue compartments exhibit significant differences in protein patterns. Estrogen stimulates high molecular weight proteins in epithelial cells, while in the stroma, low molecular weight components are most prevalent. Proteins from the epithelial compartment also show significant isoelectric charge trails suggesting the possibility of modifications in protein structure. Certain uterine proteins on the gels are being identified by antibody localization. Recent studies have suggested the possibility that steroid receptors may be phosphorylated proteins. Two dimensional gel electrophoresis was used to analyze estrogen receptor which was partially purified from mouse uteri by steroid affinity chromatography. Preparations were identified by silver staining as well as blotting on to nitrocellulose paper. Preparations were immunoblotted with ^{125}I estrogen receptor antibody. Fluorographs of these blots produced 2 spots at 70,000 mw at two different isoelectric points (pI 7.4 and 6.4). These two receptor specific spots may represent the nuclear and cytosolic forms of the receptor and possibly the more acidic component may be a phosphorylated form of the estrogen receptor.

B. PLANS FOR FUTURE

When the estrogen receptor is activated it has an increased affinity for DNA. This property is believed to explain its nuclear localization and interactive nature with the genome. Earlier results with the DES compounds have indicated weak biological activity even with significant nuclear receptor levels. It is possible that the nuclear receptor complexes produced by these compounds are not properly activated for stimulation of responses. Plans are to analyze receptor complexes bound to DES compounds for their ability to bind to DNA cellulose. A competitive displacement assay with unlabeled DES compound receptor complexes will be developed. These compounds will also be synthesized in radioactive form in order to directly evaluate the interaction. Such information should help determine what chemical structure of the ligand is involved with receptor activation. Estradiol receptor complexes bind preferentially to oligonucleotides with oligo dT having the greatest affinity. It will be possible, particularly with radioactive compounds, to measure receptor binding with the oligonucleotides to see if differences may be observed which suggest that particular ligand receptor complexes show a binding selectivity. Another plan is to test different DES isomer receptor complexes for their ability to bind nuclear acceptor protein (NAP) extracted from target tissue chromatin. It would be expected these two approaches should be correlative since they both involve analyzing the ability of estrogen receptor to interact with various genomic components, although it will be interesting to see if some complexes bind DNA but not chromatin sites or vice versa. If DES compound receptor complexes do not bind as well as the DES complex, then this could be an explanation for their poor biological activity.

Due to their chemical structures, the indenestrol compounds possess a single chiral carbon atom and, therefore, exist individually as a racemic mixture of enantiomers. A chirally-active HPLC column matrix will be used to separate the individual enantiomers of IA. Receptor binding of the individual IA enantiomers will be tested to see if one enantiomer binds preferentially over the other. Enantiomeric receptor differences may also be reflected in the nuclear receptor levels in which the active enantiomer shows a translocation of receptor the same as DES while the other enantiomer has weak activity. These studies with the individual enantiomers would demonstrate estrogen receptor chiral recognition for nonsteroidal compounds and may help explain the poor uterine responsiveness and nuclear receptor occupancy of the indanyl compounds. Indenestrol A is an in vivo and in vitro DES metabolite; these metabolites will be isolated and analyzed to determine which enantiomer is produced. IA can be produced as an in vitro metabolite by incubation of DES with horseradish peroxidase. This in vitro metabolite will be isolated to determine if the same enantiomeric form is produced under both experimental conditions. Evaluation of the enantiomeric forms will indicate if the in vivo metabolism of DES to IA produces an enantiomeric form which is hormonally active or inactive. The other indenestrol isomers, IB and ethyl indenestrol (EI), will be isolated and purified. These compounds differ from IA by having a different chiral carbon atom (IB), and EI has a chiral ethyl group rather than a methyl, as in IA. Receptor binding and nuclear receptor interactions will be measured

with these enantiomers as with the IA enantiomers. IB is more active than IA in stimulating hormonal responses and nuclear receptor occupancy; it will be interesting to assess if the IB enantiomers show receptor binding and nuclear occupancy differences.

Ultimately, we plan to isolate the enantiomeric forms of these compounds as dense atom derivatives (bromoacetoxy) so that the absolute configurations of these enantiomers can be deduced by x-ray crystallography. These data in combination with the receptor binding analysis should aid in constructing a structural basis for the receptor ligand binding site. Due to their differential in vivo properties, the DES compounds will be tested in an estrogen responsive cell culture system, such as MCF-7, to assess directly in vitro whether these response differences can be demonstrated. This test system can also be used as another means of testing any potential antihormonal activities of these DES compounds. Certain compounds (e.g., IA enantiomer or E- ψ DES) appear to have much poorer biological activity than receptor binding affinity and, therefore, would be potentially good probes for understanding steroid hormone mechanisms.

In the reproductive tract, DES has been shown to stimulate a uterine peroxidase enzyme activity. Interestingly, DES is a substrate for this enzyme system producing a DES p-quinone intermediate which rearranges to a Z,Z-dienestrol product. This DES quinone is a highly reactive compound which interacts irreversibly with DNA and protein. We plan to determine if the receptor would interact with the DES quinone. Experimental conditions will be established to support stability of the quinone in aqueous solution. Competitive binding assays and rate inhibition studies will be utilized to assess the interaction. Direct binding reactions of receptor preparations with ^3H -DES p-quinone will be used later to determine if irreversible binding is occurring. These studies would evaluate whether a potential reactive intermediate of DES metabolism in target tissue (i.e., DES p-quinone) could interact with the estrogen receptor in an irreversible manner. This type of binding could potentially produce a hormone receptor complex in vivo which is an acute persistent stimulant of tissue responses since the ligand could not dissociate from the receptor.

Studies will be continued to characterize the second nuclear receptor peak. Receptor isolated at the times of both receptor peaks will be analyzed for salt resistant binding to demonstrate tight genomic binding reactions occurring at this later peak. Such resistant sites are expected if the peak is related to genomic stimulation of tissue responses. Nuclear matrix is postulated as the intranuclear site of DNA synthesis and active gene transcription. As a working hypothesis, we will analyze nuclear matrix binding during tissue stimulation and determine if it increases at the time of the second peak. If this second receptor event is related to uterine DNA synthesis stimulation, then it is expected that a major portion of the second peak should be associated with the matrix. Later studies would attempt to characterize the chromatin by fractionation techniques at the two different peaks to assess whether new or different sites are being exposed and if these findings may be an explanation for the appearance of the second receptor peak.

In the rodent, it is not certain during development when estrogen tissue responsiveness occurs and whether the estrogen receptor is present and involved in the mechanism of action. In order to evaluate whether DES exposure and fetotoxicity of the reproductive tract is mediated through the estrogen receptor, studies are also planned to determine if the estrogen receptor is present in fetal reproductive tract tissue at the time (9-16 days) used for DES exposure. The levels of nuclear and cytosolic forms of the estrogen receptor in fetal mouse reproductive tracts will be assayed. These tissues will be analyzed by a biochemical microreceptor binding assay as well as immunohistochemical procedures using a monoclonal antibody to the estrogen receptor provided by Abbott Laboratories.

Protein and genomic markers are being sought as an endpoint of estrogen uterine responsiveness especially related to tissue growth. Proteins will be labeled and analyzed by two dimensional gel electrophoresis at early and late periods of hormone stimulation at times just prior to and during DNA synthesis. The analysis will be applied to samples from estradiol and estriol treated animals where patterns should differ since estriol stimulation induces all uterine responses but the later ones related to hyperplasia. Gels will be analyzed by computer scanning and matched for detection of proteins in the estradiol group not present with estriol. Similar analyses will be performed after uterine stimulation with the DES compounds since their varied biological activity should show differential patterns. Future plans would be to isolate any unique spots and produce antibodies which could be used for assays of the material as well as probes for a uterine cDNA library being developed in Dr. C. T. Teng's laboratory.

In conjunction with these protein gel studies, the uterine estrogen receptor will be localized in tissue samples in 2-D gels by immunoblotting and reaction with labeled estrogen receptor monoclonal antibody. Multiple forms of the receptor, which have already been demonstrated, will be tested by ^{32}P labeling to see if they represent a phosphorylated form. Experimentally, this will require making enriched or partially purified preparations of estrogen receptor by ammonium sulfate precipitation, heparin-sepharose chromatography and/or DES-dithiopropyl sepharose affinity column. Once these different receptor forms are determined, preparations will be made from tissue fractions at different points of stimulation in order to evaluate the level of phosphorylated and unphosphorylated forms. Such findings will allow the correlation of phosphorylated receptor forms to tissue stimulation. Mechanistically, EGF and its receptor specifically phosphorylate tyrosine residues of intracellular proteins. Attempts will be made to correlate uterine EGF actions, estrogen activity, and their specificity by determining whether EGF is involved in phosphorylating the estrogen receptor.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70067-02 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Molecular Mechanism of Steroid Hormone in Sex Organ Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Christina T. Teng

Others:	B. T. Pentecost	Visiting Associate	LRDT NIEHS
	J. A. McLachlan	Head, Devel. Endo. and Pharm. Section	LRDT NIEHS
	R. P. DiAugustine	Research Chemist	LRDT NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Developmental Endocrinology and Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.5

PROFESSIONAL

2.0

OTHER

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The mouse uterus has provided a system for study of estrogen action since it contains estrogen receptors and depends on estrogen stimulation for maintenance of physiological functions. We have previously identified an estrogen-stimulated mouse uterine secretory protein ($M_r \sim 70 \times 10^3$) by in vitro ^{35}S -methionine labeling experiments. Currently, the HPLC purified 70K protein was further characterized. Total amino acid analysis did not show an unusual composition. The 70K protein is a glycoprotein with an apparent asparagine-linked carbohydrate moiety. Individual sugar analysis revealed a single carbohydrate chain which contains sialic acid, galatose, mannose, fucose, and glucosamine. The NH_2 terminus of the 70K protein was cleaved with cyanogen bromide, and the resulting fragments were separated by HPLC. Two of the fragments yielded amino acid sequence. We have obtained the 32 mer synthetic oligonucleotides according to the amino acid sequence which will be used as the probe to select bacteria clones containing the cDNA insert coding for the 70K protein mRNA. Rabbit polyclonal antibody raised against the purified 70K protein demonstrated specificity for the 70K protein by "Western Blot" analysis. The uterine 70K protein was induced by estrogen but not by testosterone or progesterone. Slot blot analysis and the immuno-enzyme-linked method was used to examine the tissue distribution of the 70K protein. Tissues such as lung, brain, spleen, ovary, kidney, liver, muscle and intestine of estrogen-treated mice did not have measurable amounts of 70K protein. Only uterine and vaginal tissue gave positive reactions.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

One of the most interesting and yet poorly understood problems in developmental biology is the expression of sets of hormone-regulated genes in tissue specific ways. For example, the vitellogenin genes are transcriptionally regulated by estrogen in chicken liver cells and, yet, are totally unresponsive to the same stimulation in the oviduct despite the presence of functional estrogen receptors which mediated the expression of the ovalbumin gene. Although the gene sequences are the same in all tissue, differential expression of the gene seems associated with the modification of gene structure. Transcriptionally-active genes are largely confined to chromatin domains in which the DNA structure is in a relatively open configuration. Modulation of chromatin structure of a specific gene to this open configuration has been observed during the process of development stimulation by hormones, and integration of virus. The DNA methylation pattern of an active gene is altered from that of the inactive gene and sequential changes in DNA methylation pattern have been observed during development. Whether a hormone-regulated gene(s) acquires the capability to respond to hormone through such modification of gene structure during development needs to be investigated. An androgen and an estrogen-regulated gene in the genital tract of the male and female mouse, respectively, will be used as a model system for such studies. The morphological and physiological changes of the mouse genital tract during normal and abnormal development have been well-studied. In addition, the availability of many inbred mouse strains and the wide range of their susceptibility to steroid-hormone induced abnormal development and cancer has been reported. With this model system, we are able to gain more insight into the problem of gene expression and development as well as how prenatal toxicity will affect future gene expression. Some specific questions can be asked, such as: What gene sequences are required for hormone regulated expression? What are the events associated with differentiation-dependent changes of the chromatin structure? How does prenatal toxicity effect the ability of the genes to synthesize its product? Is there any DNA rearrangement, deletion or amplification which occurs in and around genes due to prenatal DES or exposure to other carcinogens.

Objectives

The research goal of this laboratory is to understand the expression of a steroid hormone regulated gene(s) in normal and abnormal development and to understand how this gene acquires the capability to respond to steroid hormones.

Experimental Approach, Scientific Justification, Recent Accomplishments and Significance

To approach the goal of this laboratory, our immediate task is to search for an androgen and an estrogen-regulated gene(s) in the genital tract of the male and female mouse, respectively.

1. Studies on Androgen Regulated Seminal Vesicle Secretion (SVS) IV Gene

a. SVS IV Gene of the Rat

The androgen-dependent seminal vesicle secretion IV gene from rat has been previously cloned and sequenced. The 5'-flanking region of this gene contains a perfect inverted repeat at -117 bp from the CAP site. The potential for forming a true cruciform structure in this region can be demonstrated in supercoiled configuration. Therefore, by S_1 -nuclease digestion of supercoiled plasmid pSV3.3 (containing SVS IV), we have shown that there is an S_1 -nuclease-sensitive site near the putative cruciform structure. We also detected a S_1 -nuclease-sensitive site in the 3'-flanking region of SVS IV about 800 bp from the termination site of the transcription unit. Recently, we have studied the S_1 -nuclease-sensitive and DNase I-hypersensitive sites in the chromatin of the SVS IV gene. Seminal vesicle nuclei from adult male rats were isolated by the standard heavy sucrose sedimentation method. The purified nuclei were digested with either DNase I or S_1 nuclease. The DNA was then extracted and purified with the phenol:chloroform method. The purified DNA was digested with restriction enzymes, separated on agarose gel, and transferred to a nitrocellulose filter for Southern analysis. The hypersensitive sites and S_1 -nuclease sensitive sites on the chromatin were mapped by indirect endlabeling techniques. We found that the region around -100 to -150 bp was most sensitive to DNase I and S_1 -nuclease digestion. There were several minor nuclease sensitive sites located about 5Kb upstream from the CAP site. DNase I-hypersensitive sites and S_1 nuclease sensitive sites of SVS IV gene were not detected in chromatin from rat liver in which the gene is repressed. SVS IV gene is undermethylated in the seminal vesicle and is highly methylated in the liver. It appears that the upstream DNA of an actively transcribed gene is organized in the chromatin in such a way that discrete regions become exposed and accessible to the exogenous nuclease. Similarly, these relatively open regions in the chromatin also may be accessible to regulatory molecules such as hormone-receptor complexes and RNA polymerases in the transcriptional apparatus. Our present findings correlate well with the current hypothesis that the presence of DNase I-hypersensitive sites is necessary for transcription by RNA polymerase II in vivo, and the actively expressed gene is generally undermethylated.

b. Isolation of Mouse SVS IV cDNA With Rat SVS IV cDNA Probe

In order to isolate mouse SVS IV cDNA clone with rat SVS IV cDNA as a probe, we first examined the degree of cross hybridization between rat cDNA and mouse mRNA. The size of SVS IV mRNA in the seminal vesicle of both rat and mouse were determined by RNA blot analysis. A single RNA species was identified for both the rat and mouse seminal vesicle, it was 650 base pairs in length. There are 30% cross hybridizations between rat SVS IV cDNA and mouse SVS IV mRNA. By using rat SVS IV cDNA as a probe, we have screened a cDNA library made from mouse seminal vesicle A⁺mRNA. Ten positive signals were detected among 600 recombinant clones. Plasmid DNA from one of the positive clones, pmSV16, was purified through CsCl-propidium di-iodide centrifugation. The cDNA insert of the pmSV16 was recovered by restriction enzyme digestion and agarose gel separation. The mRNA corresponding to the clone cDNA from the mouse seminal vesicle was identified by the RNA blot analysis, a 650 base pair length mRNA was hybridized with a mouse cDNA probe, and the intensity of hybridization between mouse mRNA and mouse cDNA probe was eight times stronger than hybridization between mouse mRNA and rat cDNA. In this study, pmSV16 cDNA insert was prepared for subsequent DNA sequencing. The mouse genomic library will be screened with the pmSV16 cDNA insert for mouse SVS IV genes.

2. Studies on Estrogen Regulation of 70K Protein From Mouse Uterus

a. Demonstration of Estrogen Stimulation

Mouse uterine luminal fluid obtained from estrogen-stimulated mice contains many proteins when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. One of the major proteins appeared at a molecular weight of 70,000 daltons. When estrogen-stimulated uteri were incubated with [³⁵S]-methionine in vitro, and the proteins in both incubation medium and tissue homogenate were analyzed by SDS-PAGE and fluorogram, the 70K protein was the major newly synthesized mouse uterine protein which was labeled and released into the incubation medium but not in the tissue homogenate. The ³⁵S-labeled 70K protein was not detectable in the incubation medium when the uteri were not stimulated with estrogen. Results from these experiments demonstrated that the synthesis of a 70K protein in mouse uteri was regulated by estrogen.

b. Purification and Chemical Characterization of 70K Protein

The 70K protein has been purified to homogeneity by a CM-Affi-Gel Blue column chromatography and reverse phase HPLC. Amino acid composition of the 70K protein shows 20% of aspartic and glutamic acid and 15% of lysine and arginine. The NH₂-terminal amino acid is pyroglutamic acid which appears to be blocked for sequencing. We, therefore, cleaved the 70K protein with cyanogen bromide (CNBr) and separated the resulting peptides by HPLC. Five peptides were identified. We have partially

sequenced the two larger peptides; one yielded sequence information and the other has some heterogeneity at certain positions. The fragments from CNBr treatment were also analyzed by SDS-PAGE. Five peptides were demonstrated with silver stain; they appeared at the regions of 42K, 38K, 30K, 24K, and 14K. The 42K and 38K fragments may be the result of partial digestion.

The 70K protein is a glycoprotein having an asparagine-linked carbohydrate moiety. Total neutral sugar content determined by the phenol-sulfuric acid method has been found to be about 3% sugar. The individual sugar analysis shows a single carbohydrate chain which contains sialic acid, galactose, mannose, fructose and glucosamine. The presence of carbohydrate in this protein may contribute to the microheterogeneity of the protein which appears as a multiple form with pI 9.8-10 on two-dimensional gel electrophoresis.

c. Antibody Production

Rabbit polyclonal antibody against pure 70K protein has been obtained. The specificity of the antiserum was tested on double immunodiffusion plate (Ouchterlony) and immunoblot (Western) analysis. The double immunodiffusion pattern shows one precipitin line between the anti-70K serum and the 70K protein. There was no interaction between serum proteins and the anti-70K serum. For the immunoblot analysis, total uterine luminal fluid protein and other test proteins were separated on SDS-PAGE and electrophoretically transferred from the gel to a nitrocellulose filter. The immobilized proteins were interacted with the anti-70K serum followed by the binding of a second antibody labeled with horseradish peroxidase. The location of the 70K protein was demonstrated by the immunoenzyme reaction based on the deposition of a colored enzymatic product on the nitrocellulose paper. Only 70K protein interacts with the antibody, although the duplicated filter stain with India ink shows that all the proteins were transferred and immobilized on the nitrocellulose filter.

A dot-immunobinding assay has been used to examine the tissue distribution of the 70K protein. The results indicated that kidney and liver were negative; whereas the vagina and uterus gave positive reactions.

d. Demonstration of 70K Protein Secretion From Mouse Uterine Epithelium Cell Culture

Mouse uterine epithelial cells were separated from other cell types by a combination of trypsin digestion and gentle shaking of the mouse uterus. The epithelial cells were further purified by Percoll gradient centrifugation. Purified mouse uterine epithelial cells were cultured in serum-free medium for three days. 70K protein secreted into the culture medium was determined by SDS-PAGE and Western blot. Coomassie blue stain of the SDS-PAGE revealed proteins secreted by the epithelial cell as well as the protein added to the serum-free medium such as EGF

and transferrin. A prominent protein band appeared at 70,000 dalton. Western blot analysis demonstrated that this protein gave positive reaction with 70K antiserum, no other proteins from the culture medium interacted with the anti-70K serum.

e. Isolation of cDNA Clone Coding for the Message of the 70K Protein

Antibody produced against purified 70K protein will be used for the identification of relevant cDNA clones. The antibody could be used to immunoprecipitate polysomes carrying the message and nascent polypeptide chains, or it may be used to detect clones in a total uterine tissue cDNA library prepared in an expression vector. Currently, we have constructed a cDNA library in the phage expression vector λ gt11. The ds cDNA was constructed by the "nick translation" technique pioneered by Okayama and Berg and subsequently modified for oligo dT priming.

The other approach we have chosen to isolate the 70K protein cDNA clone is to use synthetic oligonucleotide. We have synthesized a mixture of 32mer oligo-nucleotide according to the limited known amino acid sequence of the 70K protein. This oligo-nucleotide will be used as the probe to screen a general cDNA library constructed in a plasmid and transformed to the host bacteria.

B. PLANS FOR FUTURE

The research plan for the next five years will be focused on the following two major areas.

1. Studies of the SVS IV and 70K Protein Gene Expression During Normal and Abnormal Development
 - a. Currently, in our laboratory, we have established the radioimmunoassay (RIA) for 70K protein. The RIA will be performed with the liquid phase double antibody method. The ontogeny of 70K protein and its response to estrogen stimulation in mouse uterus will be investigated. Whether prenatal DES exposure could upset such a developmental process or affect its future hormone responsiveness will be examined.
 - b. Localization of 70K protein in various mouse tissue at different developmental stages of the uterus and in the uterus after estrogen stimulation will be performed by an immunocytochemical method.
 - c. The mRNA levels of SVS IV in seminal vesicle and 70K protein in the uterus will be measured by dot blot and Northern blot. How prenatal DES affects the ability of these genes to synthesize this product will be evaluated.

- d. Study of steroid hormone action in vitro. A primary epithelium cell culture system for the seminal vesicle of the male mice and the uterus of the female mice in chemically-defined serum-free medium has been established in the laboratory. Modulation of the SVS IV gene and 70K protein gene expression in primary culture system by various steroid hormones and their antagonists will be investigated.

2. Studies of the Gene Structure of SVS IV and 70K Protein

- a. Isolation and characterization of mouse SVS IV gene and 70K protein gene. The cDNA probe obtained in this laboratory for these genes will be used to screen the mouse genomic library. Nucleotide sequences and restriction map of these genomic clones will be determined.
- b. Identification of androgen receptor and estrogen receptor binding sequences of the SVS IV gene and 70K protein gene, respectively, will be carried out with nuclease footprinting and nitrocellulose filter assays.
- c. Study the methylation pattern of these genes during development. The presence of methylation in specific gene regions can be analyzed by restriction enzymes that are blocked in their cleaving activity by methylation of cytosine residue within their recognition sequences. Restriction endonuclease isoschizomer MspI and HpaII will be used to map the C-C-G-G site and Hha I the G-C-G-C site. The relationship between DNA methylation pattern and gene expression during development will be analyzed.
- d. Mapping the DNase I-hypersensitive sites. Appearance of DNase I-hypersensitive sites in and around the genes of SVS IV and 70K protein during development and after hormone stimulation will be mapped with indirect endlabeling technique. The relationship among DNA methylation pattern, appearance of DNase I-hypersensitive sites and gene expression will be correlated.
- e. Examination of prenatal DES effect on specific gene structure. DNA methylation patterns and DNase I-hypersensitive sites of the SVS IV gene and 70K protein gene from prenatal DES-exposed animals will be investigated.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 70069-03 LRDT																				
PERIOD COVERED October 1, 1984 to September 30, 1985																						
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Role of Peptide Growth Factors in Reproduction and Development																						
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 30%;">R. P. DiAugustine</td> <td style="width: 40%;">Research Chemist</td> <td style="width: 20%;">LRDT NIEHS</td> </tr> <tr> <td>Others:</td> <td>Y. Tomooka</td> <td>Visiting Fellow</td> <td>LRDT NIEHS</td> </tr> <tr> <td></td> <td>J. A. McLachlan</td> <td>Head, Devel. Endo. and Pharm. Section</td> <td>LRDT NIEHS</td> </tr> <tr> <td></td> <td>K. S. Korach</td> <td>Research Endocrinologist</td> <td>LRDT NIEHS</td> </tr> <tr> <td></td> <td>C. T. Teng</td> <td>Expert</td> <td>LRDT NIEHS</td> </tr> </table>			PI:	R. P. DiAugustine	Research Chemist	LRDT NIEHS	Others:	Y. Tomooka	Visiting Fellow	LRDT NIEHS		J. A. McLachlan	Head, Devel. Endo. and Pharm. Section	LRDT NIEHS		K. S. Korach	Research Endocrinologist	LRDT NIEHS		C. T. Teng	Expert	LRDT NIEHS
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	C. T. Teng	Expert	LRDT NIEHS																			
COOPERATING UNITS (if any) University of North Carolina (Chapel Hill) Chiron Corporation																						
LAB/BRANCH Laboratory of Reproductive and Developmental Toxicology SECTION Developmental Endocrinology and Pharmacology Section INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																						
TOTAL MAN-YEARS 2.5	PROFESSIONAL 2.0	OTHER 0.5																				
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																						
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Earlier studies in this laboratory have shown that an ¹²⁵I-labeled highly purified form of epidermal growth factor (EGF) did not cross the transplacental barrier when given intravenously to pregnant CD-1 mice. This finding supports that a complementary ligand for embryonic/fetal tissue EGF receptors may originate locally or intrinsically in the embryo. We are presently investigating this possibility with specific immunoassays for EGF and transforming growth factor (αTGF), a polypeptide with essentially identical biological properties to EGF. In another study we are investigating reproductive tract tissues and kidney for the presence of proEGF. Pronounced localization of EGF immunoreactivity occurred in mouse kidney distal tubule cells only after treatment of the sections with pronase; this finding was compatible with the localization demonstrated by other investigators with ³²P-cDNA probe for preproEGF mRNA by <u>in situ</u> hybridization experiments. We have demonstrated preproEGF mRNA by dot-blot hybridization in immature mouse uterus and in uteri of estrogen-treated ovariectomized mice. A pronase-dependent EGF-like immunoreactivity was also found in the luminal or epithelial region of uterine sections. We are currently exploring methods to extract and quantitate proEGF and ultimately determine what biological signals stimulate processing of this protein, especially in reproductive tract tissues. </p>																						

PROJECT DESCRIPTION

I. RESEARCH PROJECT

Embryonic/Fetal Epidermal Growth Factor

Nature of Problem

It is well known that EGF can stimulate the proliferation of a wide variety of cells of ectodermal and mesodermal origin in vitro. Previous studies have also shown that EGF can: (1) exhibit specific binding in vitro to several mouse embryonic tissues and also membranes from the placenta and amnion; (2) stimulate down-regulation of embryonic cell EGF receptors; and (3) stimulate incorporation of ^3H -thymidine into DNA of several tissues of mouse embryos in vitro. A mitogenic effect of EGF has also been shown for a variety of murine embryonic cell lines. The above studies support that EGF receptors form early in development and that EGF--or a related polypeptide--has a role in embryonic/fetal growth. The source of an embryonic EGF-like peptide may be placental transfer of maternally-derived blood-borne polypeptide or synthesis by embryonic or placental tissues. Previous studies in this laboratory has shown that blood-borne ^{125}I -EGF is substantially degraded by the placenta and that the intact polypeptide cannot enter fetal or embryonic circulation when administered intravenously to pregnant CD-1 mice. Reports from other laboratories have indicated that a polypeptide is present in mouse embryo extracts that has EGF radioreceptor activity but negligible EGF immunoreactivity. It was proposed that this polypeptide may be related to α -transforming growth factor (αTGF) on the basis that material with receptor activity was able to produce the transformation phenotype in a selected clone of normal rat kidney fibroblasts. TGF has been isolated and purified in another laboratory from the serum-free medium of sarcoma virus transformed embryonic fibroblasts. Amino acid sequence of TGF reveals strong structural similarities with EGF but enough sequence disparity to account for low or negligible cross-immunoreactivity with EGF.

Objective

To develop a quantitative method for measuring αTGF (TGF-1) in neoplastic and embryonic tissues.

Experimental Approach and Scientific Justification

The very low levels of apparent TGF in mouse embryos led us to design a radioimmunoassay for a direct and sensitive determination of this polypeptide. To accomplish this, the 1-15 amino-terminal region of mouse αTGF , or Val-Val-Ser-His-Phe-Asn-Lys-Cys-Pro-Asp-Ser-His-Thr-Gln-Tyramide, was custom synthesized and coupled through the free -SH to concanavalin A with the heterobifunctional reagent N-succinimidyl 3-(2-pyridyldithio) propionate and used as an antigen. Formation of the adduct was confirmed by treating the final product with dithiothreitol to yield the free TGF fragment.

Recent Accomplishments and Significance

At least two of the six polyclonal antisera raised against TGF(1-15)NH₂-ConA were effective in binding to the radioligand ¹²⁵I-TGF(1-15)NH₂. Competition of binding was demonstrated with the dialyzed lyophilized conditioned medium from cell culture of Moloney sarcoma virus transformed 3T3 cells. EGF and NGF did not compete in the radioimmunoassay. We are presently testing intact mouse and human αTGF for cross-reactivity. However, these polypeptides are available in limited amounts and presently cannot be used routinely as standards for displacement curves.

Plans for Future

The development of a RIA for αTGF should, then, provide a means for determining any relationship of embryonic αTGF levels to development and a means for examining the presence of this polypeptide in adult normal and neoplastic tissues. We also propose to examine the effectiveness of these antisera in inhibiting the growth *in vitro* of transformed cells with and without receptors for [¹²⁵I]-EGF or [¹²⁵I]-αTGF, and also compare the levels of αTGF in quiescent versus rapidly dividing normal cell populations. αTGF might be formed in regenerating livers or estrogen-treated uteri as examples. Stimulation of cell division could occur through an autocrine mechanism by the polypeptide binding to "EGF" receptors.

II. RESEARCH PROJECT

Putative Role of EGF in Mediating Estrogen-Induced Proliferation of Uterine Epithelial Cells.

Nature of Problem

It is well known that 17β-estradiol (E₂) and synthetic estrogens can stimulate in vivo proliferation of epithelial cells of the mouse uterus, mammary gland, and vagina. In collaboration with several investigators, various experimental approaches have been designed or are currently in progress to explore the notion that the mitogenic action of estrogens may occur as a result of local (autocrine or paracrine) production of a growth factor such as EGF. The findings of earlier studies strongly suggest that it is the availability of EGF and not its receptor that limits the biological response of this polypeptide in vivo. In this context, reproductive steroids have been reported to significantly affect the synthesis or levels of EGF in some organs. For example, testosterone-induced stimulation of EGF levels in male and female mouse submaxillary glands is well documented. In another study, EGF levels in immature mouse uteri were markedly increased after treatment with 17β-estradiol for seven days. It is conceivable that whether an estrogen or androgen stimulates synthesis of EGF will relate to the specific organ, rather than sex of an animal. It is also well known that EGF stimulates growth in vitro of epithelial cells derived from various steroid-responsive reproductive tract tissues.

Perhaps an estrogen-induced stimulation of EGF formation can occur in female reproductive tract tissues and accessory organs and relate causally to the cyclical proliferation of epithelial cells.

Objectives

The studies in progress described below represent an effort to examine mouse uterine tissue for potential de novo synthesis and mitogenic action of EGF and the relationship of estradiol to uterine EGF levels. These studies constitute most of my current research activities.

Experimental Approach, Scientific Justification, Recent Accomplishments, and Significance

(1) Cell culture studies. It is well known that stimulation of cell growth in vitro by EGF requires the presence of complementary high-affinity receptors on the cell surface. Although the presence of EGF receptors does not prove per se that EGF mediates growth in vivo for a particular cell population, we felt that it was important at the outset to demonstrate EGF receptors and EGF-stimulated growth of uterine epithelial cells before we propose any putative role for EGF in estrogen-stimulated growth in vivo.

We have evidence that shows specific, saturable ($\sim 50 \times 10^3$ /cell), high-affinity binding ($K_D \sim 1 \text{ nM}$) of $[^{125}\text{I}]\text{-}\alpha\text{-EGF}$ to primary cultures of mouse uterine epithelial cells prepared by Dr. Tomooka. These findings complement his studies which show that EGF in a concentration-dependent manner stimulates proliferation of these cells in vitro especially when the cells are grown on collagen. Other known growth factors did not replace EGF in maintaining growth. We are extending these studies to investigate the potential for E_2 to stimulate EGF levels in uterine and mammary epithelial cells in vitro. In addition, both cells and medium will be examined for EGF, αTGF , and somatomedin C.

(2) Immunohistochemistry. In collaboration with Dr. Peter Petrusz at UNC, the antibody-bridge technique was used along with a high-affinity rabbit antiserum to mEGF. Appropriate controls were made to discount non-specific staining or a false positive occurring as a consequence of tissue peroxidase. Sections of uteri from adult ovariectomized (OVX) and immature female mice treated with estrogen exhibited immunostaining localized at the apical region or surface of epithelial cells. Staining required treatment of sections with pronase and was markedly reduced by adding excess mEGF. Uterine sections from untreated immature females and OVX adults did not reveal significant staining. In accord with these findings, uterine luminal fluid and residual tissue obtained from diethylstilbestrol-treated immature female mice contained significant heat- and acid-stable EGF immunoreactivity.

We intend to extend these studies by examining uterine tissue for localization of (pre)proEGF (12-13) with antisera directed to various (non-EGF)

regions of this precursor. We shall also attempt to localize staining with antisera to EGF-specific arginine esteropeptidase which may be non-covalently associated with the precursor and activated by estrogens in uterine tissue. Earlier reports indicated that the esteropeptidase, as well as EGF, were significantly elevated in the submaxillary glands of immature male mice following treatment with testosterone. When non-dissociative conditions are used to isolate EGF from the submaxillary gland, the polypeptide is recovered as "2+2" complex (M.W. $\sim 70 \times 10^3$) with the esteropeptidase (14-15).

(3) In vivo studies. In these experiments, the potential for steroids to elevate the content of mouse uterine EGF is being studied. A single injection of E_2 (2 μ g) was made intraperitoneally into adult ovariectomized (OVX) CD-1 female mice. At various times up to 1 h following injection, the mice (5 per time point) were killed and the uteri quickly removed and minced together in 1 ml 0.05 M acetic acid prechilled to 4°C. The uteri were homogenized with a Polytron and the homogenate and washings (2 x 1 ml) were combined, heated at 90°C for 10 min, and frozen at -20°C. The supernatants recovered after ultracentrifugation of the thawed samples were lyophilized and reconstituted to 0.3-0.4 ml in 0.1 M PO_4 buffer, 0.1% albumin, pH 7.4, and aliquots analyzed for EGF by RIA. Extracts obtained from mice OVX 8 days that received E_2 revealed a peak (0.2-1.0 ng) of EGF immunoreactivity (EGF-ir) 1 to 15 min following treatment. By 30 min, the EGF-ir returned to control levels (< 0.05 ng). Generally, two or more peaks of activity occurred in the time course period. The peak material gave a displacement curve in the RIA parallel to that for α -EGF and also had essentially the same elution volume as [125 I] iodo- α -EGF by Sephadex G-50 gel filtration. Mice OVX 11 days generally gave a much weaker response than those OVX for the shorter periods. Liver, kidney, or adrenal EGF-ir was not altered by E_2 throughout the time course.

The very rapid and pulsatile pattern of increase of uterine EGF-ir by E_2 suggests that this growth factor can be elaborated independently of de novo protein synthesis provided that the tissue is still influenced by prior exposure to estrogen. It remains to be determined whether this action of E_2 is specific or "receptor" mediated. The hormone may trigger formation of EGF by binding to complementary receptors in the uterus which may, in turn, stimulate processing of an EGF precursor which may not be detectable by conventional EGF immunoassay.

We intend to extend these studies to examine other steroid and non-steroid estrogens and androgens for in vivo formation of EGF. In addition, we plan to examine the potential for steroids and EGF-specific arginine esteropeptidase to elaborate EGF in uterine and mammary organ and cell cultures.

(4) Dot blot hybridization. The cDNA coding for mouse submaxillary gland preproEGF mRNA was obtained from Dr. Graeme Bell of Chiron Corp. A plasmid, pmegf 10, which contains the region coding for EGF sequence will be used for hybridization experiments on nitrocellulose paper. In collaboration with Dr. Christina Teng at NIEHS, we propose to examine the mouse

uterus and other selected tissues for stimulation of EGF mRNA following estrogen treatment. So far, hybridization experiments with the ^{32}P -egf cDNA probe indicate that estrogens may stimulate EGF mRNA levels in immature mouse uteri. Northern blot analysis of A⁺mRNA from estrogen-stimulated immature mouse uteri revealed a single species of mRNA at 4.9 kilobases in size that hybridized with the EGF cDNA probe. However, the levels of mRNA for the precursor were much lower than that in the male mouse submaxillary gland or kidney. We shall extend these studies to adult OVX control and estrogen-treated animals and examine particularly the temporal pattern associated with any elevation of preproEGF mRNA caused by estrogen treatment. Our preliminary data indicate that estrogen-induced elevation of this uterine mRNA is a much delayed event in comparison to the rate of EGF elaborated in estrogen-treated animals that have been OVX for short periods, i.e. 7-11 days. Thus, the de novo formation of new precursor mRNA per se may not be required for estrogen-induced growth in short-term OVX animals. Adequate levels of precursor mRNA may be sustained by low levels of E₂, as occurs in short-term OVX animals or during diestrus; it may be the surge in E₂, as occurs in proestrus, that may "signal" processing of the EGF precursor. In long-term OVX animals, "priming" with E₂ may be important before the acute response of E₂ can be elicited. During "priming," restoration of precursor levels may occur.

Plans for Future

The findings of an earlier study suggest that a (pre)proEGF may be present in some mouse tissues, e.g., kidney. This notion is supported by the fact that a relatively high level of preproEGF mRNA is present in some tissues, despite negligible amounts of EGF-immuno-reactivity in extracts. This concept may explain why it is necessary for us to treat uterine sections with pronase in order to localize and reveal EGF immunostaining. Moreover, we were able to demonstrate pronounced staining of the distal tubule cells of the mouse kidney cortex only when the sections were first exposed to pronase; this finding is coincident with the detection of preproEGF mRNA in distal tubules by the in situ hybridization technique. We are now concentrating our efforts in developing methods to directly measure proEGF in tissues and to understand what stimuli may evoke processing of this prohormone. Stimulation of processing of proEGF may provide an autocrine mechanism for regulation of growth or transplant, for instance.

PUBLICATIONS

DiAugustine, R.P., Walker, M.P., Klapper, D.G., Grove, R.I., Willis, W.D., Harvan, D.J., and Hernandez, O.: β -Epidermal growth factor is the des-asparaginyll¹ form of the polypeptide. J. Biol. Chem. 260: 2807-2811, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70076-01 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Germ Cell-Specific Molecules of Spermatozoa

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E. M. Eddy Head, Gamete Biology Section LRDT NIEHS

Others: D. A. O'Brien Senior Staff Fellow LRDT NIEHS

K. Toshimori Visiting Fellow LRDT NIEHS

M. P. Hedger Visiting Fellow LRDT NIEHS

COOPERATING UNITS (if any)

U. of Alberta, Ontario, Canada Fred Hutchison Cancer Res. Center, Seattle

U. of Washington School of Medicine The Hospital for Sick Children, Toronto

U. of North Carolina, Chapel Hill U. of Pennsylvania School of Medicine

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Gamete Biology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

5.0

PROFESSIONAL:

3.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Monoclonal antibodies prepared to spermatozoa, spermatogenic cells, or isolated antigens are being used to identify and characterize molecules specific to germ cells. The general hypothesis being tested is that germ cell-specific gene products are responsible for the unique structural and functional characteristics of spermatozoa. Sperm components being studied are: cytoskeletal proteins and surface components. The fibrous sheath is a cytoskeletal structure which underlies the plasma membrane and surrounds the outer dense fibers and axoneme in the principal piece of the mammalian sperm flagellum. A monoclonal antibody was found to recognize a protein of apparent M_r 70 K and pI 8.5 using 2D SDS-PAGE and Western blotting procedures. Antigen appearance during spermatogenesis was analyzed by ELISA on extracts of testes from mice 16 to 30 days of age. Reactivity appeared between 18 and 20 days after birth, temporally coincident with the appearance of spermatids. The hypothesis currently being tested is that the fibrous sheath protein is a germ cell-specific intermediate filament protein of the keratin family. Studies on sperm surface components involve a glycoprotein that appears on the sperm surface during epididymal maturation. A monoclonal antibody has been used to show that the antigen is secreted by cells in the corpus epididymidis and binds to the plasma membrane of the flagellum. The antibody reacts with a glycoprotein of apparent M_r 85 K present in sperm-free fluid from the corpus and cauda epididymidis. However, detergent extraction releases an apparent M_r 54 K antigen from sperm of the cauda epididymidis, suggesting that the component is modified during attachment to sperm. The antibody reacts with proteins of apparent M_r 110 K, 86 K, and 72 K in cauda sperm extracts separated under non-denaturing PAGE conditions, suggesting that the 84 kd protein is linked by disulfide bonds to other plasma membrane-associated components.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

Spermatozoa are structurally and functionally unique cells with germ cell-specific biochemical and immunological characteristics. Most sperm components are synthesized during spermatogenesis, but post-testicular changes occur which result in loss, modification or addition of components. The sequence of events that occur during production of the morphologically distinct and fully functional spermatozoon are well described, but the specific molecules involved are not well understood. The rationale behind these studies is that it is necessary to identify germ cell-specific components, as well as to determine how, when, and where they arise, in order to understand their roles in gamete biology. Specific probes are needed to identify these components and to monitor systematically their participation in reproductive processes.

Objectives

These studies are based on the hypothesis that germ cell-specific gene products are responsible for the unique structural and functional characteristics of spermatozoa. Two important categories of sperm components currently are being studied:

(1) Sperm cytoskeletal proteins. Germ cells contain the cytoskeletal proteins actin, myosin, and tubulin, including a testis-specific alpha tubulin, but intermediate filaments have not been detected in spermatogenic cells with antisera to the major intermediate filament proteins. However, keratin-like proteins have been reported in the fibrous sheath and outer dense fibers of the sperm flagellum and in the perforatorium of the sperm head. The keratins are products of a gene family expressed in subsets in a tissue-specific fashion and are the major components of intermediate filaments. We have identified a putative keratin protein in the fibrous sheath which is first detected during spermatid development, after meiosis is completed. The objectives are to localize this and other related proteins in the sperm flagellum and head, characterize them biochemically, determine their specific time of synthesis and assembly during spermatid development, and examine their roles in gamete biology.

(2) Sperm surface components. The sperm surface is a mosaic, with most surface components restricted topographically to one of several domains on the head or tail. Antibodies can reduce fertility in vivo and inhibit gamete interaction in vitro, indicating that cell recognition and other surface events are important in these processes. Surface modifications are also known to occur during gamete formation, sperm maturation, capacitation, and fertilization. Sperm maturation antigen 4 (SMA4) is secreted by

the epididymis, attaches specifically to the membrane over the flagellum, and is shed coincident with capacitation in the female reproductive tract. The objectives are to characterize biochemically this and other surface components specific to sperm, define their distribution on the sperm surface, determine when and where they are synthesized, monitor their presence and distribution on sperm in the male and female reproductive tracts, and determine if antibodies perturb their function in reproductive processes.

Experimental Approach and Scientific Justification; Recent Accomplishments and Significance

Monoclonal antibodies are being produced by in vivo immunization or by in vitro stimulation with spermatogenic cells, sperm or isolated antigens. They are used in morphological, biochemical and physiological studies to identify, characterize and test the role of molecules specific to germ cells. Indirect immunofluorescence (IIF), immunohistochemistry, and ELISA procedures are used to define the location of antigens on isolated flagellar components or on different domains of the sperm surface, and to determine when specific antigens appear during gametogenesis. They are also used to monitor the presence and distribution of antigens during transit of sperm through the reproductive tracts and upon fertilization, to localize antigens on tissue sections, and to determine cell type and tissue specificity. One-(1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting procedures are used to estimate the relative molecular weight and isoelectric point of antigens, to examine antigen synthesis and processing during gamete formation, and to compare antigens from different cell types or species. Assays used to test the roles of germ cell specific molecules include in vitro fertilization, passive immunization, and in vitro models of capacitation and the acrosome reaction. Monoclonal antibodies which perturb these processes will be used as probes to study antigens with a putative role in reproduction.

(1) Identification and fate of a sperm tail fibrous sheath protein.

Monoclonal antibody "ATC" was found to react with permeabilized but not intact epididymal spermatozoa from mouse and rat. The antigen was detected by immunohistochemistry in late spermatids. ATC was not absorbed by the somatic tissues tested, but it did label the cytoplasm of epidermal cells in paraffin sections of mouse skin. A family of proteins of approximately $M_r=70,000$ (70 K) 68 K, 58 K, and 56 K was detected by SDS-PAGE and immunoblotting procedures, using nonionic detergent extracts of testis and sperm. These proteins were identified tentatively as keratins because ATC reacted by immunoblotting procedures with authentic keratins extracted from mouse skin. In addition, the sperm proteins have solubility properties characteristic of keratin proteins.

The fibrous sheath underlies the plasma membrane and surrounds the outer dense fibers and axoneme in the principal piece of the mammalian sperm flagellum. The IIF staining pattern suggested that ATC was binding to the fibrous sheath. This was confirmed by immunoelectron microscopy, which indicated that the antigen is distributed throughout the longitudinal

columns and circumferential ribs of the fibrous sheath. Although most sperm components are solubilized by sequential treatment in 1% Triton X-100, 0.6M KSCN, and 4M urea, in the presence of 2mM DTT, the fibrous sheath remains intact and continues to bind ATC after this treatment. However, the fibrous sheath is soluble in ionic detergent containing 40 mM DTT, and ATC reacts with a 70 K protein from both mouse and rat fibrous sheaths on 1D SDS-PAGE immunoblots. 2D SDS-PAGE immunoblots of rat sperm fibrous sheath extracts indicate that the 70 K protein has a pI between 7.5 and 8.5. The fibrous sheath has been reported previously to consist of an 80 K protein. This protein is present in fibrous sheath extracts analyzed by 1D and 2D SDS-PAGE, but only the 70 K protein is recognized on immunoblots by ATC.

Initial studies on the appearance of the fibrous sheath protein have been carried out with ELISA procedures on ionic detergent extracts of juvenile testes. Antigen is first detected in extracts of testes from day 18 mice, shortly after the first appearance of spermatids. This timing is consistent with a previous study by electron microscopy and autoradiography, which indicated that fibrous sheath assembly began during step 2 of spermatid development in the rat.

The fate of the fibrous sheath after fertilization in the mouse has been monitored by IIF with ATC. The flagellum remains visible by phase and Nomarski optics until after the first cleavage, but the fibrous sheath shows reduced antibody binding at six hours after fertilization and no binding by sixteen hours. Electron microscopy has confirmed that the fibrous sheath dissipates during this time interval. These observations indicate that while the fibrous sheath is resistant to chemical treatment, it is soluble in the egg cytoplasm.

(2) Characterization of a sperm surface component appearing in the epididymis. Monoclonal antibodies were produced by immunizing male mice with sperm from the cauda epididymidis and vas deferens of mice of the same strain. Cell lines were selected which secreted monoclonal antibodies against components on different regions of the sperm surface. In this group, antibodies were identified which did not react with most sperm until they had reached distal caput or corpus epididymides.

Appearance of new sperm surface antigens during epididymal maturation may be due to the insertion of components from the sperm cytoplasm, the exposure or modification of pre-existing surface components, or the attachment of new components to the sperm surface. Immunohistochemistry was used on sections of the male reproductive tract to determine the origin of the antigens. One of the antigens was not detected in the epididymis, two were detected in epithelial cells throughout the caput and corpus epididymidis, and one was found in epithelial cells in a short length of the distal caput epididymidis. This latter antigen, sperm maturation antigen 4 (SMA4), was present on the entire surface of the sperm tail distal to that region of the epididymis.

Sperm retained in the ductuli efferentes by ligation did not acquire SMA4, indicating that the epididymal environment was required. Absence of sperm or of testicular fluid in the epididymis after ligation did not affect production of the antigen by the epididymal epithelium. The antigen first appeared in the epididymis of mice between two and four weeks of age. It was determined by IIF that the antigen is restricted to sperm and to epithelial cells of the male reproductive tract and is not present in other tissues. The antibody induced agglutination of sperm from cauda, but not sperm from caput epididymides. These studies indicate that the antigen is secreted from a discrete zone in the epithelium of the lower caput epididymidis and attaches to a restricted domain of the sperm surface.

The nature of the association of SMA4 with the plasma membrane was examined by treating sperm with high salt, low salt, high pH, or low pH. None of these treatments caused loss of the antigen, as assayed by IIF, indicating that the antigen is attached tightly to the cell surface. The antigen is removed by treatment with low concentrations of nonionic detergent and can be detected in the supernatant by ELISA. Saccharidases failed to alter the antigen, but trypsin and papain were effective at destroying reactivity, suggesting that the determinant contains protein. SMA4 was not removed from sperm by EDTA or mercaptoethanol and, thus, probably is not attached to the surface via divalent cation-dependent or disulfide-bond-containing moieties. The antigen was still present on sperm residing in the uterus up to 12 hours but was not on sperm associated with eggs or cumulus masses flushed from the oviducts 5-1/2 hours after insemination, indicating that the antigen is lost, modified, or masked in the upper part of the female tract. Under in vitro capacitation conditions, approximately 90% of the sperm were negative by IIF by 3 hours. The loss was not inhibited by lack of energy sources or blockage of oxidative phosphorylation, but it was inhibited by low temperature, lack of protein in the medium or presence of mouse or rat epididymal fluid. Although SMA4 is not present in the rat, rat as well as mouse epididymal fluid appears to contain a factor which stabilizes binding of SMA4 to the sperm flagellar membrane. Enzyme inhibitors failed to block loss of the antigen in the absence of epididymal fluid.

Initial biochemical characterization of SMA4 indicates that the antigenic determinant is carried on a 54 K component, extracted from cauda epididymal sperm and identified by SDS-PAGE and immunoblotting procedures. The molecule does not stain with Coomassie brilliant blue but is PAS positive, suggesting that it is a highly glycosylated protein. However, on immunoblots of sperm-free fluid from the corpus and cauda epididymidis, the antigen is an 80 K component, suggesting that SMA4 in epididymal fluid is modified during attachment to sperm.

B. FUTURE PLANS

Characterization of Sperm Cytoskeletal Proteins.

Some of the proteins of the fibrous sheath, outer dense fibers and perforatorium have been partially characterized, but there is little information available on the synthesis and assembly of these structures. Also, the biochemical data currently available are inadequate to determine if some of these proteins are members of the keratin family, and if so, whether they are products of genes uniquely active in germ cells. The keratin proteins in other cell types vary in size and pI but often show immunological cross-reactivity because of conserved sequences.

(1) Synthesis of 70 K protein. In addition to the 70 K protein, ATC reacted with other proteins on 1D SDS-PAGE immunoblots of extracts of adult testis. These might be artifacts of proteolysis of 70 K protein, or similar proteins from germ cells or somatic cells of the testis. Protease inhibitors, in addition to the PMSF used before, will be added during extraction to test the first possibility. The other possibility is being examined on enzymatically dissociated germ cells from 16- to 30-day-old mice by SDS-PAGE and immunoblotting procedures. This will determine whether other proteins recognized by the antibody are present in germ cells in pachytene through middle spermatid stages of spermatogenesis and, if so, when they appear in relation to the 70 K protein. To determine the synthetic pattern of the 70 K and other proteins, pulse-chase studies will be carried out with short term cultures of germ cells isolated at different stages. Proteins incorporating labeled amino acids will be identified by 2D SDS-PAGE and autoradiography to determine if they are modified during synthesis and assembly of the fibrous sheath components. Future studies will examine whether the mRNA for this protein is transcribed in pachytene spermatocytes and stored until after meiosis is completed or whether it first appears in spermatids.

(2) Characterization of 70 K protein. The 70 K protein from rat sperm will be further characterized by 1D peptide mapping with the Cleveland procedure and by preparing immunoblots of 1D peptide maps. Other proteins recognized by this antibody, including proteins from the testis and epidermis, will be analyzed similarly and compared to the 70 K protein to determine the degree of homology between these proteins. In addition, the 70 K proteins from mouse and rat sperm will be compared by this approach, as well as by 2D SDS-PAGE and immunoblotting, to determine if species differences are detected. Possible similarities between the 70 K protein and other keratin proteins will be examined by immunostaining blots of the 70 K protein with commercially available antisera and monoclonal antibodies against cytokeratin, keratin and intermediate filament proteins. If such antibodies react with the 70 K protein, other proteins reported to be recognized by these antibodies will be examined to determine if the 70 K protein is specific to germ cells.

(3) Proteins of outer dense fibers and perforatorium. Other monoclonal antibodies have been prepared against cytoskeletal proteins in sperm. IIF studies indicate that two bind to the fibrous sheath, several bind to the outer dense fibers, two antibodies bind to outer dense fibers and perforatorium, and one binds to all three structures. These observations support the idea that a family of closely related proteins are present in these structures. The outer dense fibers have been reported to contain six proteins from 11.5 K to 78 K in size while the perforatorium is believed to consist of one protein of approximately 13 K. The specific proteins recognized by these antibodies will be identified and their origin during spermatogenesis and possible presence in other cells will be examined. It will be determined if these are keratin proteins and, if so, their degree of homology with the 70 K protein and keratin proteins in other cells.

Characterization of Sperm Surface Components.

Surface components are modified as sperm transit the male and female reproductive tracts. Changes in sperm morphology, composition, and function occur during epididymal maturation. Caput sperm are unable to fertilize, swim effectively, or bind to the zona pellucida, while cauda sperm can perform these functions. Changes in the nature and composition of the sperm surface occur during maturation and some of the molecules involved are secreted by the epididymal epithelium. Sperm attain the "capacity" to fertilize in the female reproductive tract. Sperm surface components are removed or altered, and sperm gain the ability to undergo the acrosome reaction during capacitation. Furthermore, fertilization occurs in multiple steps, during which sperm interact with and disperse the cumulus, attach to and penetrate the zona pellucida, attach to the egg surface, and fuse with the egg plasma membrane. Antisera have been reported to inhibit most of these steps, indicating that sperm surface components are involved in the fertilization process.

(1) Attachment of SMA4. Sperm will be collected from the ductuli efferentes and initial segment of the caput epididymidis and incubated with SMA4 isolated from the epididymal fluid. Sperm will be monitored by IIF or with radiolabeled SMA4 to determine if attachment occurs in vitro. These studies will be carried out in the presence or absence of whole epididymal fluid or with homogenates of the region of the epididymis which produces SMA4. If attachment occurs, these studies should suggest whether SMA4 binds autologously to a specific acceptor site on the sperm tail surface, or whether attachment requires enzymatic or other active processes involving participation by the epithelium or luminal contents. If conditions are found which allow attachment, future experiments will use enzymes to selectively modify the sperm surface, chemical treatment to alter SMA4, and changes in pH and ionic strength of the medium to perturb attachment as an approach to the dissection of the mechanism of attachment.

(2) SMA4 Acceptor. If SMA4 binds readily to the acceptor molecule in situ, extracts of caput sperm will be separated on native and non-denaturing gels

and probed with radiolabeled SMA4 to identify the isolated acceptor molecule. If SMA4 recognizes an acceptor molecule, it will be eluted and further studied by 2D SDS-PAGE. SMA4 will be affinity purified from the epididymal fluid. The 54 K protein released from sperm spontaneously in vitro, or stripped from sperm by treatment with chaotropic salts, will also be used to see if prior attachment to the sperm surface affects in vitro binding. An alternative strategy that may be used will be to prepare an SMA4 affinity column and attempt to isolate the acceptor molecule from detergent extracts of caput sperm. The sperm will be surface radiolabeled prior to extraction to monitor binding of sperm surface components to the column. Another approach to be considered is to attach one end of a bifunctional cross-linking agent to SMA4 and allow the protein to bind to caput sperm, photoactivate to effect cross-linking, extract the sperm surface components with detergent, and then use an antibody affinity column to isolate the SMA4-acceptor molecule complex. If one of these approaches is successful, a longer range goal would be to prepare an antibody against the acceptor molecule and to use that probe to determine when the molecule is synthesized and becomes integrated into the sperm tail surface domain, and whether it is altered when SMA4 binds or is lost from the sperm surface in the female reproductive tract.

(3) SMA4 Stabilizing Factor. Epididymal fluid will be fractionated by HPLC and different fractions tested for their ability to maintain binding of SMA4 to the sperm surface. It is known that the factor is stable in solution and is non-dialyzable. Initial fractionation will be by a gel filtration and fractions will be tested for activity with an in vitro assay. The inhibition of SMA4 release from sperm will be monitored by IIF and ELISA procedures. Subsequent purifications will use chromatofocusing to separate epididymal fluid components according to charge. If an active fraction can be isolated and partially purified, it will be characterized by 2D SDS-PAGE. Future studies will attempt to develop a monoclonal antibody against the isolated factor. It will be used to determine the site of origin of the factor and to examine its function. If the sperm surface acceptor molecule for SMA4 is identified and these two molecules can be shown to bind in vitro, future studies will examine how the factor stabilizes this binding.

Other related collaborative projects are being continued. They include: (1) efforts to identify maturation-dependent surface changes which give sperm from the cauda epididymidis the ability to bind to the zona pellucida, (2) detecting Leydig cell-specific surface antigens that can be used to identify unambiguously these cells, (3) testing the role of sperm surface components by in vitro fertilization in the presence of monoclonal antibodies, (4) examining the synthesis and distribution of a testis-specific glycolipid and a plasma membrane protein with which it associates, and (5) isolating primordial germ cells, using monoclonal antibodies and fluorescence activated cell sorting, to examine gene activity and replication in totipotent cells.

PUBLICATIONS

Eddy, E.M., Muller, C.H., and Lingwood, C.A.: Preparation of monoclonal antibody to sulfoxylactoglycerolipid by in vitro immunization with a glycolipid-glass conjugate. J. Immunol. Methods (In Press).

Eddy, E.M., Vernon, R.B., Muller, C.H., Hahnel, A.C., and Fenderson, B.A.: Immunodissection of sperm surface modifications during epididymal maturation. Am. J. Anat. (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70078-02 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of Stage-Specific Surface Antigens During Mouse Spermatogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. A. O'Brien Senior Staff Fellow LRDT NIEHS

Others: E. M. Eddy Head, Gamete Biology Section LRDT NIEHS

COOPERATING UNITS (if any)

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University of Washington School of Medicine
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LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Gamete Biology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.0

PROFESSIONAL

1.0

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

During spermatogenesis, regional specialization of the plasma membrane occurs, resulting in a mature gamete with distinctly polarized functional and biochemical properties. One feature of this differentiative process is the appearance of novel surface constituents in a precise temporal sequence. Both immunological and biochemical techniques have been used to characterize germ cell-specific constituents and monitor membrane assembly. Central to these studies are methods for the purification of germ cells at defined stages of spermatogenesis by enzymatic dissociation of adult or prepuberal testes followed by unit gravity sedimentation. Three areas of research have been pursued: (a) Polyclonal and monoclonal antibodies have been used to characterize macromolecules first appearing on the surface of pachytene spermatocytes, coincident with a period of maximal protein synthesis. These constituents are not shared by most somatic cells and include at least ten proteins, a probable lipid constituent, and large lactosaminoglycans. Some of these components are retained on sperm, restricted to distinct domains on the cell surface. Monoclonal antibodies have also been raised which recognize germ cell cytoplasmic antigens including an acrosomal constituent and potential cytoskeletal elements. (b) Conditions for the short term culture of adult and prepuberal spermatogenic cells have been refined to facilitate metabolic studies and the development of in vitro functional assays. (c) Protein synthesis in isolated spermatogenic cells has been examined by 2D PAGE and autoradiography following short term culture with [35 S]methionine. Synthetic profiles become more complex throughout meiosis. A number of proteins previously identified as surface antigens are synthesized in a stage-specific manner. Germ cell surface constituents exhibiting both tissue and stage specificity are candidates for further studies exploring cell-cell interactions during spermatogenesis and fertilization.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

Mammalian spermatogenesis involves the transformation of diploid spermatogonia into highly specialized haploid spermatozoa. Features of this developmental process include continuous remodeling of the plasma membrane concomitant with marked changes in cell size and shape, the appearance of a number of germ cell-specific surface molecules, and regional specialization of the plasma membrane resulting in a mature gamete with distinctly polarized functional and biochemical surface properties. Biochemical and immunological evidence indicates that germ cell-specific surface constituents are inserted into the plasma membrane in a precise temporal sequence during spermatogenesis. A number of differentiation antigens are first detected on the surface during the pachytene stage of meiosis, when the diploid genome is active and protein synthesis is maximal, while others first appear during the haploid spermatid stages.

Although few of these surface antigens have been well characterized biochemically and precise roles have been determined for none, there are three reasons why they may be important. First, they may be involved in the regulation of spermatogenesis through communication with Sertoli cells. Such molecules could function as receptors for growth factors or hormones, mediators of compartment-specific or stage-specific attachment to Sertoli cells, or effectors of germ cell translocation from the basal to adluminal compartments of the seminiferous epithelium. Second, they may function in post-testicular processes by establishing or maintaining the mosaic sperm surface, serving as sites for attachment of new moieties during epididymal maturation, being altered or shed during capacitation, or participating in recognition and attachment processes during fertilization. Third, they may represent products of genes that are active either in a cell type-specific or a stage-specific manner during differentiation of the germ cell lineage. The goals of this project are to identify and characterize germ cell surface constituents displaying tissue and stage specificity, determine patterns of synthesis and metabolic processing of these molecules, and define their functional roles either within the seminiferous epithelium or in events leading to fertilization.

Objectives:

These studies are based on the hypothesis that stage-specific surface antigens are involved in the regulation of spermatogenesis, function in post-testicular processes, and are the products of cell type-specific and stage-specific genes.

(1) Identify and characterize surface molecules that are specific to germ cells and may also be restricted to defined stages of spermatogenesis. Such molecules will be identified by preparing monoclonal antibodies to surface antigens of spermatogenic cells. These specific probes will be used in morphological studies to determine the distribution of the antigens in the testis, in purified populations of isolated spermatogenic cells, and on particular domains of the sperm surface. The antibodies will also be used in biochemical studies to define the nature of the antigens.

(2) Examine the synthesis of germ cell surface antigens. The temporal sequence of synthesis, metabolic processing and subsequent appearance of specific antigens on the surface of spermatogenic cells will be monitored using antibodies and radiolabeled precursors. These studies involve the separation of spermatogenic cells from adult and prepuberal testes using sedimentation velocity at unit gravity (2), short-term culture of these isolated germ cells with [35 S]methionine or labeled sugars, and two-dimensional (2D) polyacrylamide gel electrophoresis and autoradiography. To test the possibility of extrinsic regulation of these processes, germ cells will be cultured in the presence or absence of Sertoli cells, hormones and growth factors, or in medium conditioned by germ cells from other stages of spermatogenesis.

(3) Assess the roles of germ cell-specific surface antigens during spermatogenesis and events leading to fertilization. Cellular interactions within the testis will be monitored using co-cultures of germ cells and Sertoli cells to determine the effects of antibodies on adhesion between these cells. In vitro assays of the acrosome reaction, capacitation and fertilization will be used to evaluate the roles of surface antigens in post-testicular processes.

Experimental Approach and Scientific Justification; Recent Accomplishments and Significance

(1) Identification and initial characterization of stage-specific antigens during spermatogenesis. Germ cell surface antigens exhibiting both stage and tissue specificity have been identified by immunoblot analysis using polyclonal antisera prepared against mouse germ cells at defined stages of spermatogenesis. Four antibodies prepared previously against distinct spermatogenic cell populations recognized surface antigens on pachytene spermatocytes and germ cells at subsequent stages of differentiation, but bound to different domains on the sperm surface. These antisera were used to immunostain nitrocellulose blots from 1D and 2D SDS gels of germ cell plasma membranes. Three antibodies against pachytene spermatocytes (AP), round spermatids (ARS), and mixed germ cells (ASC) recognize a similar subset of high molecular weight antigens, plus a number of minor constituents which vary qualitatively and quantitatively between antisera. An antiserum against vas deferens sperm (AVDS) exhibits the least complex binding pattern, but recognizes some of the same antigens as the other antisera. These results suggest that at least ten surface constituents, each recognized by one or more polyclonal antisera, may contribute to the

observed stage-specific surface reactivity. Furthermore, 2D immunoblot comparisons of plasma membranes isolated from late pachytene spermatocytes and round spermatids reveal differences between surface antigens detectable at these two spermatogenic stages.

Antisera prepared against spermatogenic cell populations isolated from prepuberal mice have also been utilized to examine transitions in surface constituents during the early stages of meiosis. An IgG antibody raised against a population of leptotene and zygotene spermatocytes (ALZ) binds by indirect immunofluorescence (IIF) to the surface of early pachytene spermatocytes and germ cells at subsequent stages of differentiation. Stage-specific antigens recognized by ALZ, including both protein and probable lipid, have been localized on immunoblots from SDS gels. A dithiothreitol-sensitive 39,000 M_r (39 K) constituent has been identified as the major protein antigen that is present in early meiotic cells but absent in spermatogonia from 8-day-old (8d) animals. This antigen is present on immunoblots of preleptotene, leptotene/zygotene, and early pachytene stage spermatocytes. These results indicate that the 39 K constituent may be synthesized during pre-pachytene stages of meiosis, but not incorporated into the plasma membrane until the early pachytene stage of spermatogenesis.

Surface carbohydrate antigens are also expressed in a stage-specific manner during spermatogenesis. In a collaborative study, three lactosaminoglycans have been identified and partially characterized using monoclonal antibodies J1, C6, and A5. Antigens recognized by these antibodies are first detected by IIF on pachytene spermatocytes isolated from 17-day-old (17d) mice, the same stage where ALZ surface reactivity appears. C6 and A5 antigens are also detected on earlier meiotic stages following neuraminidase treatment, suggesting that changes in carbohydrate moieties may be an important feature of germ cell surface differentiation. Cumulative evidence from this and other laboratories suggests that the pachytene stage of meiosis, a period of active RNA and protein synthesis, represents a major transitional stage for plasma membrane reorganization during spermatogenesis.

An acrosomal constituent has been identified with monoclonal antibody 1D4, raised in BALB/cJ females against CD-1 mouse spermatogenic cell membranes. ELISA and IIF results suggest that the 1D4 determinant is restricted to haploid spermatogenic stages and is common to at least three mammalian species (mouse, rabbit, and guinea pig). Preliminary immunoblot analyses of mouse spermatogenic cell proteins indicate that the major 1D4 antigen is an ~ 85,000 M_r protein with a pI of ~ 5.9.

(2) Short-term cultures of prepuberal spermatogenic cells. A series of experiments was performed to define conditions for the short-term culture of spermatogenic cells from adult and prepuberal mice. Pachytene spermatocytes and round spermatids from adults can be cultured with > 90% viability for at least 24 hr in Eagle's MEM supplemented with 10% fetal bovine serum (FBS), 6 mM lactate, 1 mM pyruvate. In contrast, cell

suspensions from 17d testes, containing meiotic germ cells and Sertoli cells, decline to 60-70% viability when cultured under identical conditions. Viability of 17d germ cells is not improved in DMEM, F12, DMEM/F12, RPMI-1640, 199, or STIM-9, but omitting FBS and supplementing with growth factors and hormones is beneficial. TK, an enriched MEM medium without serum developed by Tres and Kierszenbaum for rat testicular cultures, results in viabilities near 80% for 17d cells cultured for 16-20 hr. Constituents of this medium include insulin, transferrin, glutamine, sodium pyruvate, retinol, growth hormone, epidermal growth factor, and testosterone. The requirement for this medium is quite age dependent; cells from 20d mice resemble those from adults, with viabilities remaining in the 80-90% range for up to 40 hr in MEM or TK. Like 17d germ cells, those from 18d or 19d mice have higher viabilities in TK than in MEM, suggesting that hormone and growth factor requirements may change markedly during a short interval of development or spermatogenesis.

(3) Protein synthesis by isolated spermatogenic cells. Spermatogenic cells isolated from 17d and adult mice have been cultured for short periods in MEM containing [^3S]methionine. On a dpm/cell basis, mixed cell suspensions from 17 day testes (meiotic germ cells and Sertoli cells) incorporate more than twice as much precursor into TCA-precipitable material as do adult cells (late pachytene spermatocytes, spermatids, and residual bodies) over an 8 hr incubation period. Autoradiograms of 2D gels demonstrate incorporation of [^3S]methionine into > 300 proteins (M_r 20,000-150,000, pI 4.5-7.5). The majority of these proteins are detected on autoradiograms and silver stained gels of both cell populations, although quantitative and qualitative differences occur. Five purified germ cell populations (preleptotene, leptotene/zygotene, 17d pachytene, adult pachytene, and round spermatid) were also cultured for 4 hr with [^3S]methionine prior to preparation for 2D gel electrophoresis and autoradiography. These results indicate that germ cells synthesize an increasingly complex array of proteins throughout meiosis, with maximal protein synthesis at the pachytene stage.

B. FUTURE PLANS

(1) Identification and characterization of germ cell-specific antigens. Monoclonal antibodies are being prepared against surface antigens of intact germ cells from adult and 17d mice using both in vivo and in vitro immunization procedures. These antibodies will be used in biochemical and physiological studies of spermatogenic cell development and function. Antibodies are screened by ELISA and IIF on live cells to monitor surface binding on both spermatozoa and earlier spermatogenic cells isolated from adult and prepuberal mice. Based on these assays, one antibody exhibiting surface binding at multiple spermatogenic stages has been chosen for further characterization. Additional antibodies which recognize potential cytoskeletal components are also being characterized. These antibodies bind to germ cells at various stages of differentiation including spermatogonia, spermatocytes, and spermatids following fixation and

detergent extraction. As measured by indirect immunofluorescence, these antibodies cross-react with filaments in Sertoli cells. Further studies are underway to compare the reactive antigens in germ cells and Sertoli cells and to examine their tissue specificity.

Topographical localization on spermatozoa and/or stage-specificity will be used as criteria for screening the antibodies with appropriate functional assays. Antigens of interest will be further characterized by standard immunological and biochemical techniques, including 1D and 2D polyacrylamide gel electrophoresis, immunoblotting techniques, enzyme sensitivities, and lectin and sugar hapten inhibition assays for glycosylated antigens.

Other monoclonal antibodies will be prepared against germ cells at specific spermatogenic stages, plasma membrane fractions, or isolated molecules. For example, stage-specific antigens identified with polyclonal antibodies can be eluted from polyacrylamide gels and used as immunogens. Antibodies will also be prepared against Sertoli cells for use in germ cell purification and assays of germ cell-Sertoli cell interactions. It is anticipated that in the future, some of the monoclonal antibodies generated will be useful in identifying germ cell-specific genes and characterizing their expression during differentiation.

(2) Synthesis and metabolic processing of surface antigens. Studies examining protein synthesis in purified germ cell populations will focus on previously described surface molecules exhibiting stage-specificity. In addition, radiolabeled sugars will be used to assess glycosylation patterns of plasma membrane constituents during spermatogenesis. Plasma membrane isolation techniques used for adult spermatogenic cells are currently being modified to permit purification of membrane fractions from prepuberal germ cells. Using these methodologies, the synthetic profiles for individual surface constituents can be determined throughout spermatogenesis, thereby providing a means for monitoring stage-specific metabolic processes and their perturbations by experimental treatment.

Although conditions for the short-term culture of prepuberal spermatogenic cells have been developed which allow synthetic studies over an 18 hr period, higher viabilities (>90%) would be desirable. Additional factors such as lipids, glutathione, BSA, FSH, selenium and other trace elements are being tested to see if further improvements in viability can be attained for these cells. Cell separation techniques are being used to determine if particular meiotic stages exhibit distinct media requirements.

Concurrently, Sertoli cells from 17d animals are being prepared and cultured according to established procedures. Isolated spermatogenic cells will be cultured in the presence of Sertoli cells or Sertoli cell-conditioned medium to assess effects on viability. Extracellular matrix from seminiferous tubules may be included in these co-cultures because of its beneficial effects on Sertoli cell viability and morphology.

Germ cells will also be co-cultured with Sertoli cells to assess interactive effects on the synthesis of surface antigens or other marker molecules. Antigens recognized by monoclonal antibodies J1, C6, and A5 are trypsin-sensitive glycoconjugates on the surface of adult pachytene spermatocytes and round spermatids. These cells are negative by IIF after isolation with trypsin, but the C6 and A5 antigens reappear on the surface after overnight culture. In contrast, the J1 antigen is not detected after 18 hr in culture. Sertoli cells will be included in these cultures to assess their effects on J1 antigen expression.

The C6 and A5 antigens, as well as other antigens identified with polyclonal antibodies, may be present in pre-pachytene stages either within the cell or masked on the surface by sialic acid or other residues. Delayed expression of surface molecules may be a consequence of testicular architecture, with germ cell-specific antigens restricted to the late meiotic and haploid stages behind the blood testis barrier. Monoclonal antibodies in conjunction with in vitro radiolabeling procedures will allow us to monitor actual synthesis of these surface antigens and the regulation of their production in a stage-specific manner. Inhibitors of transcription, translation, and glycosylation may also be useful for examining metabolic processing steps prior to antigen appearance on the cell surface.

(3) Functional assays examining the roles of germ cell-specific surface antigens. The limited capacity of mammalian germ cells to differentiate in vitro hampers the development of bioassays monitoring spermatogenesis. However, techniques for the isolation of germ cells at major stages throughout spermatogenesis combined with improved culture conditions and monospecific probes will allow studies examining short-term interactions between testicular cells. In addition, the availability of a variety of mutant mouse strains, defective at distinct stages throughout spermatogenesis, should be helpful in determining the function of stage-specific cellular constituents.

Co-cultures of germ cells and Sertoli cells have been utilized to demonstrate differential adhesion between specific spermatogenic stages and Sertoli cells and the formation of intercellular junctions, as well as stimulatory effects of germ cells on Sertoli cell secretion of androgen binding protein. Recently, Enders and Millette have developed a co-culture system for monitoring adhesion between isolated germ cells labeled with fluorescein diacetate (pachytene spermatocytes or round spermatids) and Sertoli cells. In collaboration with these investigators, we will test monoclonal antibodies generated against both germ cells and Sertoli cells for effects on adhesion between these cells. Since earlier spermatogenic stages including spermatogonia and pre-pachytene spermatocytes exhibit surface binding with a number of available monoclonal antibodies, these cells will also be tested in the adhesion assay. Other quantitative assays measuring the forces and kinetics of cellular interactions may also be employed.

Many of the monoclonal antibodies currently being studied bind to specific domains on the sperm surface, suggesting that reactive antigens may be involved in post-testicular processes. Sperm from the caput and cauda epididymis will be compared to assess possible modification of surface antigens during epididymal transit. In vitro assays of capacitation, the acrosome reaction and fertilization will be used to monitor the modification or loss of surface antigens in the female reproductive tract and to learn if particular stages of gamete binding and recognition are perturbed in the presence of antibodies. Antigens of particular interest will be isolated and further characterized to assess their precise roles in these reproductive processes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70090-02 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Neuroendocrine and Neurochemical Regulation of Gonadal Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Negro-Vilar Head, Reprod. Neuroendocrinology Section LRDT NIEHS

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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Reproductive Neuroendocrinology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.1

PROFESSIONAL

2.1

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Reproductive Neuroendocrinology Section is carrying out studies focused primarily on the cellular and subcellular mechanisms regulating the release of luteinizing hormone-releasing hormone (LHRH) and other hypothalamic peptides that participate in the modulation of pituitary hormone release. Specific studies are designed to elucidate the role of monoaminergic neurotransmitters in the release of LHRH from nerve terminals, the nature of the specific aminergic receptors involved in the neuronal activation that precedes LHRH release, the clarification of the post-receptor events that participate in the peptide-release process, the involvement of arachidonate metabolites in amplifying the response to key neurotransmitters, and the additional role played by other intracellular messengers such as Ca^{+2} and other putative intracellular messengers derived from the metabolism of membrane phospholipids. Other parts of the project are directed to perform an in-depth analysis in vivo of the changes in monoamine turnover and metabolism in discrete brain nuclei that are known to be involved in regulation of gonadal function. Different experimental paradigms are employed, to re-create situations calling for an enhanced (or altered) function of the hypothalamic-pituitary-gonadal axis, such as steroid-feedback manipulations, pregnancy, lactation, estrous cycle, stress, ablation of selected endocrine glands or brain areas, etc. Finally, a group of experiments are directed to evaluate the mechanisms underlying the effects on the reproductive sphere of neonatal neurotoxin treatment, as well as the developmental changes and the role of steroids on certain sexually dimorphic patterns of gonadotropin secretion. The results are integrated to provide a comprehensive hypothesis of the complex, multi-level regulatory mechanisms modulating gonadal function.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

Reproductive events are regulated by highly complex neuroendocrine mechanisms and several levels of integration (hypothalamic, pituitary, gonadal, and reproductive tract) of these mechanisms are recognized. These key levels of integration, while representing very important physiological sites whereupon regulatory events take place, also constitute target areas where many hormones, regulatory factors, drugs, and toxic compounds act to affect reproductive functions. Our laboratory has worked extensively in the last several years to evaluate the different factors that interact at all levels of integration to modulate reproductive function, and to define the specific mode and site(s) of action of different agents that regulate and/or interfere with reproductive processes.

More specifically, an evaluation of the role of neural peptides and aminergic neurotransmitters on peptide hormone release and hormone action has been the primary goal of our studies. In particular, we have focused our research on the factors affecting the function of the luteinizing hormone releasing hormone (LHRH) neuronal system. LHRH is a hypothalamic decapeptide considered to be the prime physiological regulator of gonadal functions. The modulatory role played by gonadal steroids on elements affecting the LHRH neuron, particularly on central monoaminergic and peptidergic neurotransmission, has also been evaluated and the information obtained integrated with that of other paradigms to provide a comprehensive hypothesis of the role played by these different neuromodulators on reproductive events.

Objectives

The primary objectives of this research project are the following:

(1) To understand and clearly define the intimate cellular and subcellular mechanisms involved in the release and function of LHRH, particularly in relation to the sequence of events leading to enhancement of neuronal LHRH release in response to noradrenergic activation. Specific objectives to be achieved include determining the location of the norepinephrine (NE) neurons involved in LHRH release, examining the possible role and/or interaction that other monoaminergic and peptidergic (particularly opiate) systems may play on this noradrenergic stimulation, defining the nature of the adrenergic receptor(s) involved in the response, the subsequent participation of arachidonate metabolites in amplifying the response, and metabolites in amplifying the response, and the additional role played by other putative intracellular messages, such as Ca^{+2} and factors derived from the phosphatidylinositol cycle.

(2) To define the cellular interactions between steroids and central monoaminergic systems in the regulation of the LHRH neuron by exploring selective changes in amine metabolism in specific brain nuclei involved in gonadotropin regulation after removal of gonadal steroids or following replacement therapy with steroids.

(3) To analyze the characteristics of pulsatile hormone secretion, particularly that of the gonadotropins LH and FSH, and other pituitary hormones that may potentially affect central LHRH neurons, such as ACTH and β -endorphin. Since pulsatile secretory patterns can profoundly affect target organ responsiveness, a definition of the precise mechanisms governing episodic hormone release can provide much needed information on how specific neurotransmitter systems can affect pituitary or gonadal function.

(4) To explore the mechanism(s) through which selected agonistic analogs of LHRH (LHRH-A) can suppress fertility in both males and females. In particular, to study in detail the central site(s) and mechanism(s) of action of these LHRH-A adversely affecting reproductive function.

Experimental Approach and Scientific Justification; Recent Accomplishments and Significance

(1) Experiments from our laboratory have defined the neurochemical regulatory mechanisms involved in the release of LHRH and on its stimulatory action on pituitary and gonadal function. In particular, we have developed an in vitro short term culture of (hypothalamic) median eminence fragments, a brain region that contains the richest concentration of LHRH-nerve terminals in the central nervous system. It is from this region that LHRH is secreted into the hypophyseal portal circulation to reach the anterior pituitary and stimulate gonadotropin release. Since this is a closed-circuit system of difficult access, a fact which precludes the constant monitoring of changes in LHRH secretion, our method has provided much needed information on the neurochemical events underlying LHRH release under physiological or pathophysiological conditions. With the use of our in vitro median eminence culture preparation, we have ascertained that catecholamines, and norepinephrine in particular, are the key neurochemical signals that trigger release of the peptide from this area of the brain, and do so by interacting specifically with presynaptic, α -adrenergic receptors located in the median eminence region. Stimulation of these receptors leads to release of LHRH primarily through arachidonate metabolites, in particular prostaglandin- E_2 . Arachidonic acid by itself can increase LHRH release, but this effect can be nullified by inhibiting cyclo-oxygenase activity. Although other arachidonic products may also enhance LHRH release (including lipoxygenase and the newly discovered epoxygenase, P-450-dependent products), our results indicate that they are not essential for release of the peptide to occur. More recently, we have determined that phosphatidylinositol turnover may also be involved in the activation of neuronal LHRH release, since both diacylglycerol and phorbol esters can markedly enhance release of the peptide in vitro. Taken together,

these studies indicate that after interacting with a specific membrane receptor (e.g., α -adrenergic), neurotransmitters generate transmembrane signals that enhance LHRH release by mechanisms that involve multiple intracellular messengers (Ca^{2+} , inositides, prostaglandins, etc.).

(2) Very recent studies in our lab indicate that an opiate (possibly β -endorphin) neuron is part of the neuronal system governing the NE-PGE₂-LHRH interaction. A combination of in vivo and in vitro studies suggest that blockade of opiate receptors inhibits the noradrenergic neuron, allowing for an enhanced secretion of NE. This in turn induces further production of PGE₂ and the latter enhances LHRH release. This mechanism is operative in intact animals, but is not completely functional after gonadectomy. Evidence from other laboratories suggests that replacement therapy with gonadal steroids can reestablish this intriguing and prime example of a modulatory peptide-amine-steroid interaction within the central nervous system.

(3) Another important portion of our research efforts in recent years has been dedicated to the study of the neurochemical mechanisms that integrate all the environmental (external) and internal signals that the brain is exposed to during the course of a normal (or abnormal) reproductive event. In particular, our studies have characterized the role of central noradrenergic, serotonergic, and dopaminergic systems as integrators of signals (external or internal) that affect pituitary hormone secretion and gonadal function. We have looked extensively at the effects of steroids, in either physiological or pharmacological paradigms, upon the reproductive system and, in particular, within the brain-pituitary axis. Our studies have indicated that the gonadal steroids (estradiol, progesterone, testosterone) can have profound effects upon central monoaminergic and peptidergic systems involved in reproductive function. These effects were characterized during the normal course of reproductive life, and after endogenous or exogenous manipulations were introduced to modify steroid levels in plasma and target tissues. These studies indicate, for instance, that estrogens can profoundly affect brain regulation of gonadal function by interacting to a large extent with noradrenergic neurotransmitter systems within the preoptic-hypothalamic region of the brain as well as 5-HT systems in the mediobasal hypothalamus. Other steroids, such as progesterone, also can interfere with gonadal function by acting at specific brain sites, but only after specific progestin receptors are induced by prior estrogen priming. These reports have provided a regional mapping within the brain of the cellular and subcellular mechanisms involved in steroid hormone action. Recent additional studies in our laboratory have taken advantage of the information obtained in normal animals to use it in the analysis of the mechanisms mediating the adverse reproductive effects of certain neurotoxins. The data indicate that toxic neurochemicals given during the neonatal period (when the brain is still undergoing key developmental changes) can interfere with the steroid-monoamine-peptidergic neuronal interaction and thereby permanently alter normal gonadal function.

(4) Prolonged treatment with superactive agonists of LHRH suppress testicular and ovarian function in many species, including the human. The mechanism(s) and site(s) of action for these effects are not yet clearly understood. Recent studies in our Section have revealed that while exerting a distinct suppressive effect on both the endocrine and gametogenic function of the testis, LHRH-A can also have direct central actions upon brain LHRH neurons. This action of the analogs was seen also in the absence of the testis, precluding therefore any interaction with testicular products. Further studies suggest that these interactions of the analogs with the endogenous LHRH system may also involve opiate neurons. Since in the human the testicular site of action of the analogs has been found to be of minor significance, these results gain more relevance by indicating that the central actions of the LHRH-A may be an important component of the antigonadal effect of these peptides.

(5) We have characterized recently the pulsatile secretion of FSH and the selective regulation of LH and FSH pulses by gonadal steroids and peptides (inhibin). As a corollary to these experiments and in view of the fact that selective suppression of either LH or FSH can be achieved, we have now undertaken studies to determine if LHRH is involved in the regulation of both LH and FSH pulsatile secretion or whether FSH release is independently regulated.

B. FUTURE PLANS

(1) With regard to the mechanisms regulating LHRH release, additional in vitro experiments are needed in this area to clarify the following:

(a) The exact mechanism by which noradrenergic activation of α -adrenergic receptors affect prostaglandin- E_2 and LHRH release. Specific questions to be asked include whether α -1 or α -2 type receptors are involved. This, in turn, may provide a clue to indicate which of several possible intracellular mediators (i.e., Ca^{+2} , cyclic nucleotides, etc.) is more likely to be involved in the action of the catecholamine within this particular neuronal system.

(b) Transmembrane events involved in peptide release. Our studies will focus on the role of stimulated phosphatidylinositol (PI) turnover and protein phosphorylation on the release of peptides. Studies will be conducted to evaluate the possibility of measuring inositol phosphates formation as well as protein phosphorylation in our tissue preparation in vitro, after either in vivo or in vitro treatment with specific hormone secretagogues. The role of arachidonic acid and of different arachidonoyl residues in LHRH release will also be evaluated by studies employing 3H - or ^{14}C -labeled arachidonic acid in combination with specific metabolic inhibitors (indomethacin, NDGA, phospholipase A_2 inhibitors, etc).

(c) Pertaining to the previous item, we will continue our studies which have characterized the role of calcium channels and of intracellular Ca^{+2} release on the stimulation of LHRH induced by membrane depolarizing agents or by prostaglandins, respectively. Experiments will be performed to evaluate the precise role of intracellular Ca^{+2} on peptide hormone secretion. In conjunction with the studies outlined above, these observations should provide much needed information concerning the molecular mechanisms involved in the regulation of peptide release and hormone action.

(d) By using an automated, computer deliver-controlled cell perfusion system (recently incorporated to our Section), we plan to evaluate the effects of modifying stimulation parameters (frequency, amplitude, duration of pulse, etc.) of specific neurotransmitter secretagogues as well as superimposing different stimuli on the pattern of LHRH release in vitro.

(e) The information obtained from the above in vitro experiments will be integrated with available information and, where possible, compared with experiments in which in vivo cannulation of portal vessels and portal blood collection performed after selected treatments, to obtain correlative information on how accurate the in vitro system is in predicting in vivo changes in peptide release.

(2) In the area of steroid-amine-peptide interactions, our efforts will be focused on elucidating:

(a) The mechanisms through which steroids allow the opiate inhibition of NE-stimulated LHRH release to be expressed. In addition to performing the required neurochemical estimates of NE turnover and metabolism during different steroid regimens, we intend to explore the possible role of gonadal steroids in the expression of opiate receptors in brain regions involved in LHRH regulation.

(b) The exploration of the three main opiate systems (β -endorphin, enkephalins, and dynorphin) present within the preoptic-hypothalamic region in order to determine their precise role in regulation of LHRH secretion. This exploration will be accomplished by direct measurement of peptide levels in, and release from, specific hypothalamic regions and also by using available mRNA and cDNA probes to evaluate whether the expression of these opiate peptides is specifically modulated by gonadal steroids or other neuromodulators.

(3) Using the technique of portal blood cannulation, we intend to analyze by frequent sampling the characteristics of LHRH release in vivo. This would require the use of the Fink method of portal blood collection, which

allows collecting samples at very short intervals, combined with a highly sensitive and specific LHRH assay established in our laboratory. It is hoped that, if a definite pulsatile pattern can be characterized, this will allow us to replicate that pattern using our computerized perfusion system to mimic in vitro the conditions observed in vivo. If this approach proves to be successful, then it could provide the means to evaluate the role of steroids, peptides, and/or amines in modifying LHRH/LH release.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70092-02 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Cellular and Molecular Mechanisms Mediating Peptide Hormone Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Negro-Vilar Head, Reprod. Neuroendocrinology Section LRDT NIEHS

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COOPERATING UNITS (if any)

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Yale University Medical School, Department of Obstetrics and Gynecology
The Wellcome Research Laboratories, Department of Molecular Biology

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Reproductive Neuroendocrinology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.9

PROFESSIONAL

1.9

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Analysis of the cellular and molecular mechanisms mediating peptide hormone action constitute an important component of the research efforts of the Reproductive Neuroendocrinology Section. The close interrelationships mediating neuroendocrine responses within the hypothalamic-pituitary-gonadal systems offer an excellent opportunity to analyze some unique characteristics of peptide-peptide, peptide-amine, and peptide-amine-steroid interactions. Studies using pituitary cell cultures are directed to evaluate the precise mechanisms through which peptidergic or aminergic secretagogues enhance or suppress peptide hormone release. Protocols are designed to evaluate characteristics of hormone-receptor interactions, post-receptor as well as transmembrane events involved in the hormone-release process, and definition of the specific intracellular messengers transducing the action of key hypothalamic peptides involved in pituitary hormone release. Other studies are designed to explore the intriguing peptide-amine interactions (such as serotonin-AVP) previously reported by our group, to better understand the nature and physiological significance of those interactions.

At the testicular level, studies are designed to determine the intratesticular effects of LHRH-analogs, known to adversely affect both the endocrine and the gametogenic functions of the testis. Since some of these analogs are presently being tested for use in human contraception, an understanding of their site(s) and mechanisms of action is of obvious significance. The interaction of these LHRH-A with intrinsic peptidergic systems within the testis, such as the proopiomelanocortin-derived peptides, is also being explored. The results may provide very significant advances to our knowledge of paracrine and/or autocrine effects of gonadal peptides.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

Great advances have been made in recent years to further our understanding of some of the intrinsic mechanisms of hormone action. Several basic systems of intracellular messengers coupled with membrane receptors or of cytosolic receptors, which upon nuclear translocation affect the genome, have been defined in a variety of tissues. Within the hypothalamic-pituitary-gonadal system, a diversity of aminergic, peptidergic, and steroidal hormones exert profound hormonal effects by evoking a multitude of cellular responses. In each individual case, the hormone-cell interaction acquires special characteristics according to the chemical nature of the hormone involved, and to the type and location of the target cell. Most hormones exert specific effects at selected sites, and this specificity usually involves stimulation (or sometimes inhibition) of key intracellular messengers after interaction with a specific cellular receptor. Definition of the sequence and type of events mediating the effects of different peptides or amines within the hypothalamic-pituitary-gonadal axis is, therefore, of paramount importance toward our understanding the intimate cellular and subcellular mechanisms mediating major reproductive events.

Objectives

The primary objectives of this research project are the following:

(1) To evaluate and characterize the mechanism(s) and specific cellular and subcellular site(s) of action of certain key peptidergic and aminergic neurohormones known to be secreted from the hypothalamus into the portal circulation, which modulate the release of different pituitary hormones clearly involved in reproductive functions. In addition, special attention will be given to peptide-amine interactions that may well represent an important mode of combined hormonal regulation. Finally, possible paracrine and autocrine actions of peptide hormones will also be evaluated.

(2) Much evidence indicates that most hormones are secreted in a pulsatile pattern and that this episodic mode of secretion plays an important role in receptor activation and hormone response. We plan to analyze the effect that mode of stimulation (i.e., amplitude, interpulse interval, pulse height, etc.) or input deprivation (i.e., absence of pulsatile stimulation) have on peptide-induced hormone release from cultured dispersed cell systems. Attempts will be made to induce up or down regulation of peptide receptors and to evaluate the changes in intracellular messengers that may mediate those modifications in cell response.

(3) In support of the previous studies, we intend to evaluate the mechanisms underlying the enhanced cellular responsiveness at the hypothalamic

and pituitary level that is observed in adult animals after neonatal neurotoxin treatment. Elucidation of these mechanisms may provide some fundamental answers toward our understanding of the factors that regulate cell responsiveness under physiological or pathophysiological conditions.

(4) In order to explore the paracrine and/or autocrine role of peptides in selected endocrine tissues, we propose to use the testis as a model to determine the presence and characterize the identity and location, within specific compartments or cell types of the testis, of several peptidergic factors which have been recently postulated to be present in the testis of several species. These studies will, in addition, attempt to define the physiological role of these peptides, as well as the particular circumstances in which their action is clearly expressed.

Experimental Approach and Scientific Justification; Recent Accomplishments and Significance

(1) Work carried out by scientists presently at the Reproductive Neuroendocrinology Section has established and characterized specific pituitary membrane receptors for hypothalamic peptides such as angiotensin II (AII) and arginine vasopressin (AVP). In both cases, high affinity, low capacity membrane receptors were identified, with K_d 's in the low nanomolar range, values found compatible with the doses of peptide required to elicit a normal physiological response. In the case of AVP, the use of 3H -ligand enabled us to evaluate both the binding characteristics as well as the biological activity (enhanced ACTH and β -endorphin release) using intact, dispersed anterior pituitary cells. Specific receptor blockers, like saralasin, blocked the action of AII, and other agonists of either AII or AVP showed binding affinities comparable with their respective biological activities. Since other groups have shown that corticotropin releasing factor (CRF) acts also on specific membrane receptors, it is clear that three different peptides that stimulate ACTH/ β -endorphin secretion do so by acting on specific and independent receptors. Moreover, while CRF-stimulated ACTH release involves enhanced cAMP formation the effect of AVP is not mediated by activation of that nucleotide, strongly suggesting that other intracellular messengers might be involved in the action of AVP and, perhaps, of AII.

Additional studies from our group have clearly established an intriguing peptide-amine interaction between AVP and serotonin to enhance ACTH release from the pituitary. Several studies involving the use of specific serotonin receptor blockers, serotonin synthesis inhibitors, or tissues obtained from rats (Brattleboro DI) with a genetic deficiency in AVP synthesis, indicate that the AVP-serotonin interaction can be observed in vivo and might, therefore, constitute an important physiological mechanism in the regulation of ACTH release.

(2) We have recently explored the role of two specific intracellular activators of protein kinase C, diacylglycerol and a phorbol ester (phorbol 12, 13 dibutyrate; PDBu) on anterior pituitary hormone release in vitro.

Using a dispersed cell system, we established that both compounds enhance the release of all anterior pituitary hormones (ACTH, β -End, GH, TSH, PRL, LH and FSH) in a concentration-and time-dependent manner. Certain hormones, like GH, ACTH and β -End, were particularly sensitive to the actions of these secretagogues. The results suggest that stimulus-mediated activation of PI turnover may result in the intracellular formation of diacylglycerol and the subsequent activation of the Ca^{2+} activated, phospholipid dependent protein kinase C. The ensuing protein phosphorylation may be an important component of the processes leading to peptide hormone release.

(3) Recent studies in our Section have established the presence of proopiomelanocortin (POMC)- derived peptides in testicular interstitial fluid (TIF). Using highly sensitive and specific radioimmunoassays, we established the presence of β -endorphin and ACTH in TIF from both intact and hypophysectomized animals. The latter observation clearly indicates that the peptides found in TIF are not derived from the peripheral plasma, since after removal of the pituitary gland plasma levels of both POMC-peptides are undetectable. Authenticity of the peptides measured was further established by chromatography and parallelism of serial dilutions with the standard curve in the radioimmunoassays. Recent experiments indicate that stimulation with either LH or HCG both in intact or hypophysectomized animals can profoundly affect levels of these peptides in TIF suggesting a relationship between gonadotropin stimulation and the POMC-derived peptides. Quite recently, we have obtained evidence that another neural peptide, angiotensin II, is present in large concentrations in human follicular fluid. These observations, coupled with recent reports describing POMC-like peptides in gonads and reproductive tract of both sexes, as well as the demonstration of the expression of a POMC-like gene in testis and epididymis indicate local synthesis and regulation of these peptides and suggest a paracrine or autocrine role for these factors in endocrine and/or gametogenic functions.

(4) Another series of studies have been conducted to elucidate the effects of superactive luteinizing hormone-releasing hormone (LHRH) analogs on testicular function. Chronic and continuous administration of different LHRH-A has been shown to suppress both the endocrine and gametogenic functions of the testis, representing the basis for the potential use of these compounds as contraceptive agents. Studies in the Reproductive Neuroendocrinology Section have been directed at analyzing the direct intratesticular effects of these analogs of LHRH on testosterone secretion and also their possible interactions with other peptidergic systems present in the testis. Preliminary results indicate that an interaction between LHRH and POMC-derived peptides in the testis may be involved in the regulation of testosterone secretion. The results also suggest that LHRH analogs have biphasic effects upon Leydig cell function, with short-term stimulatory and long-term inhibitory effects being observed.

B. FUTURE PLANS

Several experimental approaches will be employed to evaluate peptide/amine effects at the cellular/subcellular level in the anterior pituitary. Most of the studies will continue to require the use of in vitro systems and a combination of either short-term or long-term tissue or cell cultures. In the case of the pituitary gland and due to the heterogeneity of the cell types, attempts will be made to process enzymatically dispersed cells through an elutriation procedure which may provide useful enrichment of certain cell types. In addition, cell cultures will be submitted to specific stimulation by hypothalamic peptide hormones, and attempts will be made to mimic the pulsatile rate of delivery of these neural hormones with the use of recently developed computerized cellular engineering perfusion systems.

These approaches will enable us to search for answers to the following problems:

(1) Role of certain intracellular messengers on the peptide-induced anterior pituitary hormone release. Using specific and selected peptidergic secretagogues, such as AVP, AII, CRF, LHRH, or TRH, attempts will be made to characterize the cellular events leading to the exocytotic secretion of hormones. Particular attention will be given to the possible effects of peptides on membrane phospholipids. This will be accomplished by using P-32 labeled cells to study the breakdown and resynthesis of phosphatidylinositol, by measuring activation of the phospholipid-dependent protein kinase C, and by employing certain specific probes such as diacylglycerol, phorbol esters, and ionophores. Since either inositol biphosphate (IP^2) or inositol triphosphate (IP^3) may function as messenger(s) to transmit the signal expressed by surface receptors to the interior of the cell, we will explore the possibility that one of these factors may be involved in the action of some of the above peptides. In addition and in light of recent evidence that we and others have provided indicating that arachidonic acid and/or arachidonate metabolites can enhance pituitary hormone release, experiments will be conducted to evaluate the linkage between the receptors regulating arachidonic acid turnover and the PI cycle. The role of Ca^{+2} on the above events as well as the possible direct role of arachidonic acid as a second messenger will similarly be explored.

(2) Peptide-amine and peptide-peptide interactions at the anterior pituitary level. Combined stimulation with peptides using selected pulsatile delivery patterns in a continuous perfusion system will be employed to evaluate peptide-peptide interactions. Similar approaches will be used to analyze peptide-amine interactions. Cells will be obtained from animal models in which the secretion of the appropriate pituitary hormone is selectively enhanced or suppressed or after experimental manipulations

designed to alter the in vivo input of a given peptide or amine. When appropriate, autoradiographic studies will be performed to analyze the uptake and intracellular handling of peptidergic or aminergic ligands.

The specific objectives of these experiments are to determine (a) at the physiological level, how important are these peptide-peptide and peptide-amine interactions in a given integrated biological response and (b) at the cellular and subcellular level, what intracellular systems participate in these interactions. As an example, we would ask the question: is the AVP-serotonin interaction that results in an enhanced secretion of ACTH/ β -Endorphin a physiologically relevant phenomenon? Further, is that interaction mediated by a common intracellular messenger resulting in a potentiation of the effects or is it expressed by the independent stimulation of two different messenger systems, perhaps converging later in a final common pathway?

(3) Role of peptides in testicular function. Recently developed superagonists of LHRH, currently being evaluated for clinical use in contraception, will be employed to determine the direct effects at the testicular level as well as possible indirect effects at central (hypothalamic) and pituitary levels. Specific parameters to be evaluated at the testicular level will be testosterone secretion into testicular interstitial fluid and peripheral plasma, as well as testosterone secretion from whole testis in short-term in vitro cultures. The possible interaction between LHRH-A and other testicular peptides, particularly β -endorphin and ACTH, will also be evaluated. In selected experiments, blockade of opioid receptors will be achieved by naloxone or naltrexone pretreatment to determine the role of endogenous testicular opioids on the endocrine function of the testis.

PUBLICATIONS

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70094-01 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Neuroendocrine Regulation of Prolactin and Pro-Opiomelanocortin-Derived Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. A. Johnston Senior Staff Fellow LRDT NIEHS

Others: A. Negro-Vilar Head, Reprod. Neuroendocrinology Section LRDT NIEHS

COOPERATING UNITS (if any)

University of California-San Francisco, School of Medicine

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Reproductive Neuroendocrinology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.0

OTHER

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Experiments by the Reproductive Neuroendocrinology Section focus on the identification and development of cellular and subcellular mechanisms involved in the regulation of pituitary hormone secretion, in particular, prolactin (PRL), adrenocorticotrophin (ACTH), and β -endorphin (β -end). Specific studies are designed to elucidate the role and interactions of monoaminergic neurotransmitters and peptide releasing and release-inhibiting hormones in the secretory control of these hormones from the anterior (AP) and neurointermediate (NIL) lobes of the pituitary, the sequential arrangement and possible connectivity of that neuronal circuitry, and the developmental maturation of that circuitry. Furthermore, various stressful stimuli not only dramatically affect reproductive function as well as the secretion of PRL, ACTH, and β -end, but the stress-induced increase in plasma PRL has also been shown to require a developmental maturation postnatally. Therefore, this paradigm has been utilized to selectively examine the neuronal mechanisms involved in the stress-induced increase in PRL secretion. In addition, drugs which interact selectively with specific neurotransmitter or neuropeptide neuronal systems are utilized to determine the individual contributions of specific neuronal systems to the regulatory secretion of PRL, ACTH, and β -end, and to aid in elucidating the neuronal connectivity involved in that regulation. Lastly, specific lesions and surgical procedures are employed to determine the source of various neurotransmitters and neuropeptides which are found in the pituitary or have been shown to act at the pituitary level, and the role that those neurotransmitters/neuropeptides play in the dynamic regulation of PRL, ACTH, and β -end secretion. These studies are integrated to elucidate the neuronal mechanisms, integration, and connectivity involved in the neuroendocrine regulation of the secretion of particular hormones from the pituitary which have been shown capable of affecting reproductive function.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

Monoamine neurotransmitters and neuropeptide hormones appear to play a critical role in regulating the secretion of hormones which affect reproductive function, in general, and in the neuroendocrine control of PRL, ACTH, and β -end, in specific. Elucidating the underlying neurochemical processes, as well as the individual components and interactions of the monoamines and neuropeptides involved in the neuroendocrine regulatory mechanisms governing the secretion of these hormones, is physiologically important in order to understand the sites where this neuroendocrine regulation takes place. It is also important to understand the underlying mechanisms involved in reproductive dysfunction associated with the abnormal secretion of these hormones (i.e., during chronic stress, hyperprolactinemia) as well as determining possible target areas which may be vulnerable to pharmacological attack. Our efforts are concentrated on defining the specific central mechanisms which modulate reproductive function, particularly to evaluate the mode and site(s) of action of various pharmacological agents and stressful stimuli that interact/interfere with this neuroendocrine regulation.

Objectives

To clarify the neurochemical/neuropeptide mechanisms and interactions which underlie the development and maturation of the central neuroendocrine response to stress; to define the role played by individual monoaminergic and peptidergic neuronal systems in the release of the "stress" hormones: PRL, ACTH, and β -end; and to determine the origin, control, and regulatory role of monoamines/neuropeptides which are found in the pituitary or have been shown to act at the level of the pituitary in the regulation of the secretion of PRL, ACTH, and β -end. It is hoped that uncovering the neuronal circuitry regulating the secretion of these hormones which can affect reproductive function will not only allow us to understand the basic mechanisms involved in their neuroendocrine regulation and developmental maturation but will also allow the clarification of neurochemical mechanisms which may be responsible for their aberrant secretion and consequent effects on reproductive function.

Experimental Approach and Scientific Justification and Recent Accomplishments and Significance

(1) It is well established that different stressors can adversely affect reproductive function. Furthermore, the hypersecretion of hormones which are released in response to stressful stimuli such as PRL, ACTH, and β -end have been shown to affect reproductive function. Recent studies have been directed toward the elucidation of the discrete neurochemical mechanisms

which integrate the various signals that the central nervous system receives during a stressful stimulus and result in the rapid release of PRL, ACTH, and β -end from the pituitary. A method utilizing high performance liquid chromatography combined with electrochemical detection (LCEC) has been developed in order to concurrently determine femtomol levels of monoamine neurotransmitters, their precursors and metabolites in discrete microdissected brain nuclei, peripheral and hypophyseal portal plasma, and other fluids. Plasma concentrations of PRL, ACTH, and β -end are quantitated using radioimmunoassays. The hormonal and neurochemical responses to ether stress have been analyzed in an attempt to describe the role that monoaminergic neurotransmitter systems in microdissected discrete brain nuclei play as integrators of signals that regulate the pituitary secretion of those hormones. As expected, the results demonstrate that acute exposure to ether vapors induce large increments in plasma PRL, ACTH, and β -end. Under these conditions, a detailed mapping of monoamine metabolism in several discrete areas of the hypothalamus in ether-stressed animals demonstrated increases in NE metabolism in several discrete nuclei, a differential effect on 5-HT metabolism in various discrete nuclei, and a selective increase in DA metabolism in the rostral portion of the arcuate nucleus that was temporally correlated with these hormonal changes. These results suggest that the observed changes in monoaminergic metabolism may be involved in the neuroendocrine regulatory mechanisms governing the release of these three hormones in response to stressful stimuli. These studies have also indicated that an endogenous opiate neuronal system is inhibitory toward the ether stress-induced increases in plasma ACTH and β -endorphin as well as norepinephrine metabolism in the medial preoptic hypothalamic area. That is, the ether stress-induced increases in these three parameters are all augmented by pretreatment with the opiate receptor antagonist, naloxone. Using a neurotoxin (monosodium glutamate, MSG) which is known to cause dramatic neurochemical deficits in the arcuate nucleus, we evaluated the effect of these MSG-induced deficits on the ability of ether stress to influence the secretion of PRL and ACTH. Despite the large neurochemical deficits that neonatal MSG treatment caused in discrete areas of the rat brain, the results indicate that these deficits did not qualitatively influence either the neurochemical or hormonal responses to ether stress.

The increase in plasma PRL concentrations in response to ether stress is not observed in infantile (13 day old) rats but is present in prepubertal (36 day old) and adult animals. This requisite developmental maturation is not due to an absence of the inhibitory tone that the tuberoinfundibular DA system normally exerts on PRL secretion. Furthermore, this required maturation is not a generalized phenomenon which is shared by all "stress" hormones. We have demonstrated that the ether stress-induced increases in plasma ACTH and β -end are present even in infantile animals. We utilized the selective absence of an increase in plasma PRL in response to ether stress in infantile rats to elucidate the neurochemical components involved in the ether stress-induced release of PRL, without the added complicating factor that these neurochemical changes may be related instead to the stress-induced alteration of some other hormone.

The data demonstrate that the maturation of the PRL response to ether stress is coincident with the appearance of an increased 5-HT neuronal activity in the medial basal hypothalamus (MBH) and a decreased neuronal activity of DA in the median eminence (ME) in response to ether stress. Furthermore, indirect stimulation of 5-HT neurons following the administration of the immediate amino acid precursor of 5-HT, 5-hydroxytryptophan (5-HTP), decreased DA synthesis in the ME and increased PRL secretion even in infantile animals, indicating that the inhibition of ME DA synthesis is downstream to the 5-HT neuronal activation and that this mechanism is functional even in the infantile animal.

Previous evidence has shown the necessity of intact 5-HT neuronal systems in the opiate-induced increases in PRL. Our data indicate that these events may be functionally related. Because the stress-induced increase in plasma PRL can be blocked by the administration of the opiate receptor antagonist, naloxone, we used the opiate receptor agonist, morphine, as a pharmacological tool in order to evaluate more precisely the role that opiate receptor activation plays in the ether stress-induced increases in PRL. Opiate receptor activation by morphine results in the stimulation of 5-HT neuronal activity in the MBH, decreased DA synthesis in the ME, and an increased secretion of PRL into the plasma. Thus, activation of opiate receptors can elicit the cascade of responses that result in an increased plasma PRL even in infantile animals. The functional deficit in the ether stress-induced increase in PRL secretion which requires a developmental maturation must be functionally located prior to opiate receptor activation. Either the endogenous opiate neuronal system is not activated by ether stress stimulus in the infantile animal, or once activated, the opiate system is not yet functionally connected with the opiate receptor(s).

The neuronal connectivity of this neuroendocrine response may not be as simple as it once appeared. Recent evidence demonstrates that opiate receptor blockade with naloxone administration not only prevents the ether stress- and morphine-induced increases in MBH 5-HT metabolism, decreases in ME DA synthesis and increases in plasma PRL but also prevents the 5-HTP-induced increase in plasma PRL. Our own data in conjunction with previous studies suggest a dual pathway governing PRL release in response to ether stress. One pathway contains a functional sequence of an endogenous opiate-5-HT-DA neuronal connectivity (with the possibility of interneurons at each connection) which decreases the tonic inhibition exerted by DA at the level of the anterior pituitary. The second pathway is proposed to exhibit a functional sequence of a 5-HT-endogenous opiate connection which regulates the secretion of a PRL releasing factor (PRF) which is only fully capable of enhancing PRL release in the presence of a decreased DA inhibitory tone. It would be of interest to determine whether these two proposed pathways are differentially regulated in response to stress or during other experimental paradigms where PRL undergoes a dynamic secretory change (proestrous surge, lactation/suckling, pregnancy).

Recent studies may have provided information as to the location and possible identification of the PRF proposed to play a role in the second regulatory pathway suggested above. We have recently performed experiments which demonstrate that the 5-HTP-induced increase in plasma PRL is completely abolished in animals who have had their neurointermediate pituitary lobe (NIL) removed previously. Removal of the NIL, by itself, did not alter basal PRL concentrations. The neurochemical analysis revealed that 5-HTP selectively influenced the 5-HT neuronal systems; that the increases in 5-HT neuronal activity resulting from 5-HTP administration were not sufficient, in the absence of the NIL, to increase PRL; that 5-HTP must first be enzymatically converted to 5-HT in order to exert its effect on plasma PRL; and that the required conversion of 5-HTP to 5-HT occurs at a site(s) which is susceptible to the actions of a peripheral (not CNS active) decarboxylase inhibitor. These data suggest a dual regulation and the lack of a significant role for this proposed PRF in the basal secretion of PRL which is primarily governed by tonic dopaminergic inhibition. The possibility that a factor(s) located in the NIL may be responsible, in part, for the stress-induced increase in PRL, and attempting to identify that factor(s) are topics currently being investigated.

(2) Other studies have focused on the source of various monoamine neurotransmitters which have been found in the anterior pituitary (AP) or have been shown to act directly at the pituitary level to influence hormonal secretion. Neurotransmitters which have been shown to exert direct effects on AP hormone secretion at the pituitary level include epinephrine (EPI) and 5-HT. Our studies using LCEC analysis of hypophysial portal plasma have demonstrated for the first time the presence of a high concentration of EPI in the hypophysial portal plasma, which is the vascular connection between the ME and the AP. In support of these studies, experiments were conducted in which the AP tissues were isolated from normal neural and vascular regulatory influence by surgically transplanting the AP under the renal capsule, the NIL was surgically removed, the ME was electrolytically lesioned to remove its influence, or the activity of the enzymes involved in the synthesis of these monoamine neurotransmitters were measured in the ME, AP and NIL. The results indicate that 5-HT found in the AP is not synthesized there, does not come from the NIL, and is not carried preferentially to the AP through the hypophysial portal vasculature under basal conditions. Rather, the present evidence supports the hypothesis that 5-HT in the AP is derived from blood-borne elements. On the other hand, the AP content of the metabolite of 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), appears to be at least partially transported to the AP via the hypophysial portal vasculature and is, at least in part derived from the ME. These data suggest for the first time that a compound thought of normally as an end product metabolite may actually possess a physiologically important regulatory function of its own. This possibility will be investigated in future experiments.

B. PLANS FOR FUTURE

(1) With regard to the maturation and development of the ether stress-induced neurochemical and hormonal response, additional experiments are planned to:

- (a) Apply selective lesions to cell body and/or terminal areas of neuronal pathways of the monoamines/neuropeptides thought to be involved in the regulation of PRL, ACTH, and β -end in an attempt to determine the role of these systems in the secretion of these hormones in vivo.
- (b) Use in vitro microperifusion techniques, pharmacological antagonists, and passive immunization techniques in an attempt to determine the identity of the NIL PRF responsible for the 5-HTP-induced increase in plasma PRL and the location of the proposed 5-HT-opiate-PRF interaction. When available, the effect of antagonists developed toward potential PRFs (like oxytocin) on the 5-HTP- and stress-induced increases in PRL will be determined. Similarly, when a specific antibody against a putative PRF becomes available in sufficient quantity, the effect of passive immunization of the animal against the putative PRF on the 5-HTP- and stress-induced increase in PRL will be examined. Preliminary evidence indicates oxytocin does not play a major role in the increases of PRL in response to 5-HTP, ether stress, or suckling. Finally, to evaluate whether 5-HTP stimulation of the release of PRL is the result of a direct NIL-anterior pituitary interaction, the in vitro effect of 5-HT and 5-HTP as well as candidates as PRFs (oxytocin, VIP, vasopressin, PHI-27, TRH, and several endogenous opiates) on PRL secretion from whole pituitaries (NIL plus AP) or from anterior pituitaries, alone, will be evaluated.
- (c) Recent studies by our laboratory suggest that an endogenous opiate neuronal system is inhibitory toward the ether stress-induced increases in plasma ACTH and β -end as well as norepinephrine metabolism in the medial preoptic hypothalamic area. Therefore, we intend to determine the time course of the effect of naloxone administration on the ether stress-induced increases of PRL, ACTH, and β -end in animals with indwelling jugular catheters in order to examine more precisely the exact nature of the opioid interaction with ACTH or β -end secretion as well as NE metabolism in the medial preoptic hypothalamus. Preliminary data indicate that naloxone potentiates the ether stress induced increases in ACTH and β -end and attenuates the increase in PRL for up to one hour following ether stress stimulus.
- (d) Take advantage of a genetic animal model (BS/ZTM rats, Dr. Klaus Dieter Döhler, Max Planck Institute, West Germany) which exhibits a selective deficiency in that even as adults this strain of rats

selectively fails to exhibit an increase in plasma PRL in response to stress. This rat strain will be evaluated hormonally and neurochemically in the hope that this genetic mutant will represent an animal model where the developmental maturation of the PRL response to stress did not progress past the infantile stage. If this premise promises to be true, then we can examine the model further to understand more thoroughly at a discrete, functionally important level the identity and location of the exact neurochemical correlates for this requisite maturation.

- (e) Utilize immunocytochemical methods in an attempt to determine the location of the 5-HT and opiate interactions (medio-basal hypothalamus, raphe nuclei, NIL) in adult animals as well as throughout development.

(2) With regard to the source and role of monoamines in the secretory control of AP hormones, we will:

- (a) Examine paradigms where PRL secretion is undergoing a dynamic change (i.e., proestrous surge, lactation/suckling, pregnancy or following morphine or 5-HTP administration) and evaluate monoamine metabolism in discrete hypothalamic nuclei and hypophysial portal plasma concentrations of the monoamines and metabolites in order to determine the role of these neurotransmitters in the secretion of that hormone.
- (b) Examine the effects of pharmacological antagonists and passive immunization procedures against various putative PRL releasing factors (i.e., oxytocin, vasopressin, TRH, VIP, endogenous opiates) during paradigms involving a dynamic secretion of PRL in order to understand more clearly the role of these factors in the neuroendocrine regulation of PRL during these paradigms.
- (c) Examine the possible effects of microperfusion of 5-HIAA at various concentrations on pituitary hormone secretion in an attempt to determine whether the high concentrations of that 5-HT metabolite measured in hypophysial portal plasma may play a physiological role in pituitary hormone secretion.
- (d) Evaluate the effects of selective lesions of cell body and terminal regions and surgical procedures designed to isolate the pituitary tissues from possible sources of monoaminergic supply in order to determine the source of those monoamines in their neuroendocrine regulation.

The resulting information from this dissection of the neurochemical/ neuropeptide mechanisms involved in mediating PRL, ACTH, and β -end release under basal and stressful conditions may also provide relevant information concerning the usefulness of various psychoactive compounds in the treatment

or prevention of stress-mediated events which can affect reproductive function, as well as clarify the site(s) and mechanisms of action of environmental, toxicological or pharmacological insults which profoundly affect reproductive functions.

It is hoped that understanding the neuronal circuitry regulating the secretion of these hormones which can affect reproductive function will not only allow a better understanding of the basic mechanisms involved in their neuroendocrine regulation and developmental maturation, but will also allow the clarification of neurochemical mechanisms which may be responsible for their aberrant secretion and subsequent effects on reproductive function.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70096-01 LRDT

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Pulsatile Pituitary Hormone Secretion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Michael D. Culler Senior Staff Fellow LRDT NIEHS

Others: Andrés Negro-Vilar Head, Rep. Neuroendocrinology Section LRDT NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Reproductive and Developmental Toxicology

SECTION

Reproductive Neuroendocrinology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2.0

PROFESSIONAL

1.0

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues x ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

It has recently become patently clear that the concept of "blood concentration" of a given pituitary hormone may be relatively meaningless in light of the discovery that these hormones are secreted in a rhythmic, pulsatile manner. The pattern of hormone release seems to be far more important than the quantity in determining the response of the target tissue(s). Yet despite the recognized importance of pulsatile hormone secretion, very little is known about the effects of altering the parameters (frequency, duration, shape, amplitude, etc.) of the pulsatile signal on the target response. The proposed projects examine several important aspects of pulsatile pituitary hormone secretion and will lay the groundwork for changes in the way endocrine problems are evaluated and treated. First, the pulsatile pattern of LH and FSH secretion will be characterized in freely moving, cannulated rats. An emphasis will be placed on elucidating separate control mechanisms for the pulsatile release of the two gonadotropins. Secondly, the effects of altering the parameters of pulsatile input signals on LH-FSH and ACTH- β -endorphin release from incubating pituitaries will be determined using a computer-controlled perfusion apparatus. Since we are one of the few, if not the only, laboratory in the world with this capability, our contribution to the knowledge of pulsatile hormone regulation will be quite unique. In addition, the possibility of spontaneous pituitary pulses (without external stimulation) will be examined. Thirdly, the effect of altering hormonal input signals on the intracellular messengers regulating cellular responses will be studied using the computer assisted perfusion system and monolayer pituitary cultures. In particular, the phenomena of desensitization and sensitization will be examined to elucidate the responsible intracellular messenger system(s). Information gained from these studies should be almost immediately translatable into information for improving treatments of infertility and for future prospects of fertility regulation.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem

During the last decade, it has been established that all of the anterior pituitary hormones, as well as many extrapituitary hormones, are secreted in a rhythmic, pulsatile manner. It is now patently clear that the "blood concentration" of a given hormone, as measured in a single sample, may mean relatively little compared to the pattern of its secretion and the resultant fluctuation in blood concentration over time. Recent evidence has suggested that the frequency and duration of hormone pulses may be much more important than the size or amplitude of a pulse in determining the response of the target organ(s) or system(s). Pulse frequencies that are too rapid or a constant, non-pulsatile infusion of a given hormone causes the target organ(s) to become refractory, a phenomenon known as desensitization. Conversely, certain discontinuous patterns of hormone administration or secretion can cause enhanced target organ response. Yet, despite the recognized importance of the hormone delivery pattern in determining the response of the target cells, very little is known about the effects of altering the pattern of administration for most hormones, and almost nothing is known concerning the intracellular mechanisms and requirements of the target cells in responding to a pulsatile signal.

Two of the primary hormone systems known to regulate reproductive function are the brain-pituitary-gonadal axis and the brain-pituitary-adrenal axis. In both instances, releasing hormones, which influence certain pituitary cells types, are believed to be released from hypothalamic nerve endings into the hypophyseal portal circulation in a pulsatile manner. The frequency, duration, and amplitude of the pulses are governed by the balance of physiologic input signals to the brain at a given moment. The pituitary responds by releasing the appropriate hormone in a pulsatile manner governed by the incoming hypothalamic and other peripheral signals. The target organ for the pituitary hormone then responds appropriately, signaling its status back to the brain and pituitary to further regulate release patterns and complete the hormonal circuit. Knowledge of how changing the pattern of hypothalamic signals can alter anterior pituitary function and of the intracellular pituitary events that modulate the response are of paramount importance in understanding reproductive function during a given physiologic state and essential for developing proper hormone regimens for treatment of dysfunction and control of fertility.

Objectives

The primary objectives of this project are the following:

- (1) To evaluate and characterize the pulsatile release of the gonadotropins, luteinizing hormone (LH), and follicle stimulating hormone (FSH),

from the anterior pituitary in vivo. Since only one hypothalamic releasing factor, luteinizing hormone-releasing hormone (LHRH), is known which can stimulate the release of both gonadotropins, a major concern of this project will be to determine the extent to which the two hormones are controlled through common hypothalamic pathways or are independently regulated. The manner in which various physiologic and experimental models, known to influence reproduction, alter the pattern of LH-FSH secretion will also be examined.

(2) To determine the role of input signal pattern on pituitary response in vitro. Focus will be placed on the secretion of LH-FSH and ACTH- β -endorphin and how altering the parameters of pulsatile input (amplitude, frequency, duration, shape, etc.) of the respective releasing hormones (LHRH and CRF) can influence the response. In addition, the influence of other regulatory factors will be examined and how the overlapping, interacting patterns of administration can alter secretion. Finally, the possibility will be examined that certain hormones may be released in spontaneous pulses from the pituitary and, if so, how these pulses contribute to the overall output and are influenced by the various regulatory input signals.

(3) To examine intracellular mechanisms which are influenced by and/or may mediate the response to pulsatile input signals. Emphasis will be placed on how alteration of the input signal can influence the various intracellular messengers and how this in turn is translated into an altered cellular response. The mechanisms mediating the phenomena of desensitization and sensitization are of primary interest.

Experimental Approach and Significance

In order to examine pulsatile hormone release in vivo, it is necessary to take frequent (10 minutes or less) blood samples and to avoid the use of anesthetics that can alter brain function. For this reason, unanesthetized, unrestrained rats with indwelling atrial cannules will be used for studying pulsatile hormone release under the various experimental models of this project. To evaluate the role of LHRH in controlling LH-FSH secretion, the effect of passive immunoneutralization with anti-LHRH serum on the pulsatile release of both will be examined. If divergent control mechanisms can be established, then further studies will examine the contribution of other active factors in gonadotropin regulation such as follicular fluid inhibin, sex steroids, etc. If possible, the role of the newly discovered GnRH-associated peptide (GAP), which may be selective for FSH secretion, will also be examined. Knowledge gained concerning the pattern of LH-FSH secretion in vivo under various physiologic states will be used as a partial basis for the in vitro experiments comprising another portion of this project.

The in vitro experiments to determine the effect of altering the parameters of pulsatile releasing hormone administration on pituitary response will be carried out using a computer controlled perfusion system recently acquired

by our lab. The use of computer control with this system makes it possible to simultaneously control the delivery of up to 5 input signals to the incubating pituitary glands or cells. This system provides control of pulse, height, shape, duration, frequency, and amplitude, as well as the ability to alter these parameters during the course of an experiment. Two examples of planned experiments achievable only with this system are: (1) following the establishment of a stable LHRH pulse delivery pattern which can maintain the LH release response from incubated pituitaries, have pulse frequency gradually increase to mimic the postulated in vivo effect following sex steroid withdrawal and (2) examination of the interaction of the many ACTH releasing factors (AVP, AII, oxytocin, 5-HT, CRF, etc.) when administered in various overlapping pulsatile configurations and in various combinations. Attempts will be made to replicate pituitary pulse sequences, observed in the previously described in vivo studies, in order to determine the sequence and combination of events resulting in specific pulse patterns. This information can then be used to design further in vivo studies to create a truly integrated study system.

Examination of intracellular events mediating responses to specific patterns of signal input will also necessitate the use of in vitro systems. To this end, we will again utilize the computer assisted perfusion system as well as a static monolayer pituitary cell culture system. While pulsatile input cannot be mimicked in the static culture system, certain intracellular studies require the immediate accessibility to the cells and the large number of replicates within a given experiment which are obtainable only with a cell culture system. One of the primary objectives in studying pulsatile hormone release is to understand the events mediating the phenomena of desensitization and sensitization. Using the static cell culture system, working models of these phenomena will be established and used to determine the involvement of the various intracellular systems, such as receptor-adenylate cyclase coupling, cAMP production, Ca^{++} flux, polyphosphoinositol turnover, prostaglandin synthesis and release, etc. Models of intracellular events worked out with the cell culture system will then be tested with the computer assisted perfusion system to verify the effects of appropriate and inappropriate signal input.

Taken collectively, the outlined objectives and experimental approach form a tightly integrated study network to provide information on how the brain regulates pituitary hormone secretion by modifying the various parameters of input signaling and how the pituitary receives these messages and responds. Knowledge gained from the study of divergent LH-FSH regulation and of the effect of signal alteration on LH-FSH release will be almost immediately translatable into invaluable information for improving treatments of infertility and for future prospects of fertility control. The use of computer assisted perfusion, by providing an in vitro model of the ever changing environment of input signals found in vivo, will allow a gradual dissection of the contribution and significance of each signal regulating pituitary secretion and will result in a much greater understanding of the regulation of the system as a whole. At the present time we are one of the only laboratories in the world with the technical sophistication

gained through computer assisted perfusion and as such will be able to make a very unique contribution to the understanding of pulsatile hormone signaling. Finally, understanding the intracellular events underlying desensitization and sensitization may provide the reason for the necessity of pulsatile signaling and how these signals must be altered in order to alter cellular function. This information could provide insight into the treatment of not only certain forms of infertility but endocrine dysfunction in general and provide the basis for the design of novel hormonal treatment regimens.

Recent Accomplishments and Significances

(1) Through the use of passive immunoneutralization with high titre ovine anti-LHRH serum in two week castrate rats, we have observed that LH secretion can be suppressed dramatically within 1 hour (84% suppression) and will remain low throughout 48 hours while FSH secretion very gradually falls with time, reaching only a 54% suppression by 24 hours. In chronically cannulated, unanesthetized, unrestrained animals, passive LHRH immunoneutralization causes an immediate cessation of LH pulses and a gradual lowering of mean plasma FSH levels but has no significant effect on either FSH pulse frequency or amplitude. Injection of sufficient synthetic LHRH to overcome the antiserum blockade results in a single "burst" release of LH similar to an endogenous pulse, while pulsatile FSH is relative unaffected. The mean plasma levels of FSH, however, are restored to their original elevated level by the LHRH treatment. Collectively, these results are highly significant as they provide very strong evidence that: (a) LHRH regulates pulsatile LH secretion but not that of FSH, and (b) LHRH seems to act in concert with another, as of yet unknown, factor which is responsible for pulsatile FSH secretion. Ever since the isolation of LHRH more than fifteen years ago, it has been widely assumed that LHRH controlled both LH and FSH secretion. Since LH and FSH control very different reproductive functions in both the male and female, establishing that they are regulated by separate releasing factors will greatly change the thinking concerning development of treatment regimens for dysfunctions involving the gonadotropins.

(2) Since obtaining a computer assisted perfusion apparatus in December 1984, we have made significant progress in validating its use for pulsatile hormone studies. Pituitaries are kept viable in six individual chambers through controlled temperature, gas, pH, etc. They are responsive to LHRH and CRF for up to 12 hours (the maximum time tested so far) in a dose-related manner. Through the use of a mathematical model to predict peptide mass in the culture chamber at any given interval, pulses are designed for total mass rather than concentration per time point. By measuring both the releasing hormone used to generate the input signal and the responding pituitary hormone in the same collected samples (after passing through the chamber), it is apparent that the response follows the same basic configuration as the input signal: a rapid rise to a peak level followed by a gradual decline as the signal is washed out. By selecting the parameters of an approximately 15 ng pulse of LHRH every 40 minutes, a stable pattern

of LH release can be achieved. This basic stabilized regimen will be used as the basis for determining the effect of altering the parameters of the input signal in future experiments. A highly significant finding is that after several initial pulses of CRF, the releasing hormone stimulating ACTH/ β -endorphin release, the pituitaries continue to release fairly regular pulses of ACTH/ β -endorphin spontaneously. This finding is of particular interest from the standpoint of intracellular regulation of pulsatile hormone secretion.

(3) We have successfully established a static pituitary cell culture system which is sensitive and responsive to LHRH and CRF in a dose-related manner. Using this system, we have developed a model for studying desensitization by exposing the cells to fresh media daily which contains sufficient CRF to induce a stimulatory effect. After 3 days, the cells become refractory to CRF stimulation. If the CRF is removed for 24 hours, the cells regain full responsiveness to the peptide. Recently, we have discovered that following desensitization to CRF, the cells are also refractory to stimulation of ACTH release with AVP, AII, and 5-HT. This is particularly significant since these releasing agents are all believed to act through different receptors and intracellular messengers. The common desensitization, however, indicates a common intracellular mediator at some point. By comparing the intracellular messengers evoked by each compound in this model, a great deal of insight can be gained as to the mechanism of desensitization of endocrine tissues.

B. PLANS FOR FUTURE

(1) To examine possible mechanisms controlling FSH secretion in vivo. Using the passive LHRH immunoneutralization model used to establish divergent regulation of pulsatile FSH-LH secretion, the effect of various substances known to influence gonadotropin secretion (such as ovarian inhibin, sex steroids, etc.) can now be selectively tested for their effects on pulsatile FSH release. Of particular interest is the recently discovered GAP peptide that represents a portion of the pro-LHRH molecule. Since this molecule seems to have a higher percentage of FSH releasing activity than LH releasing activity, passive immunoneutralization with anti-GAP serum may provide selective suppression of pulsatile FSH. Regulation of FSH may also be further examined through a combination of immunoneutralization and selective brain lesions of areas believed to be involved with FSH regulation.

(2) By building on the model of stable LH release already established with the computer driven perfusion system, the effect of altering pulse frequency, amplitude, and duration on subsequent pituitary responsiveness will be examined. In addition to studies simply comparing the effect of "changed" parameters, experiments will examine the effect of ongoing changes in pulse parameters such as a pulse frequency which changes from 1 pulse/40 minutes to 1 pulse/10 minutes over a 12 hour period. The effect of superimposing pulsatile delivery of two or more factors regulating ACTH

release will also be examined. While interaction of these factors has been extensively examined in static systems, there is no information as to how that interaction manifests itself in a dynamic situation such as is found in vivo. Finally, further studies will be made to extend our knowledge of the observed spontaneous ACTH pulses in vitro.

(3) The recently developed model for CRF desensitization, using the static cell culture system, will be used to examine the effects of desensitization on the various intracellular messenger systems to determine the involvement of each. Intracellular systems stimulated by the various ACTH releasing ligands such as the cAMP production system, Ca^{++} flux, PI turnover, arachidonic acid metabolic pathways, etc., will be examined to determine if they are altered by CRF desensitization. The results from these studies will be used to devise models of intracellular desensitization which will then be tested using the computer assisted perfusion apparatus. In addition, a model for sensitized pituitary responses will be developed based on peptide deprivation strategy. The involvement of intracellular messengers in this phenomena will also be examined.

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COMPARATIVE MEDICINE BRANCH

COMPARATIVE MEDICINE BRANCH
Summary Statement

Administrative Activities

NIEHS animal facilities underwent a triennial site visit for accreditation by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Reaccreditation in full was granted following changes in animal observation practices during off-duty hours. This request by AAALAC reflects increased stringency in their general policies rather than a change in existing NIEHS practices. AAALAC remains the only recognized animal care facility accreditation body in the United States. Accreditation is an increasingly important status for private and public research facilities throughout the U.S., and is a specific goal for all NIH institutions.

Remodeling of the Bldg. 15 facility to accommodate Laboratory of Behavioral and Neurological Toxicology LBNT laboratories was completed in May 1985. Remodeling and re-equipping of the Bldg. 15 Animal Facility Service area was completed at the same time. Completion of this project was delayed 5-6 months beyond the anticipated completion date, thus complicating management problems.

During 1984 a work assessment study was conducted in the Animal Husbandry Section by outside professional services to determine acceptable work loads for animal care personnel, to evaluate the distribution of work, and to provide a basis for cost assignment for animal care service. The findings of this study, together with the previous study conducted a year earlier on the Glassware/Media Service, were used as the basis for assigning costs of these services to using Laboratories/Branches according to proportionate use. While this procedure does not result in exchange of funds, a basis for program service use measurement was established. Monthly status reports to Lab/Branch Chiefs provide accounting information to meter progress and resource utilization. It is understood that "budgets" for these services cannot be exceeded by Lab/Branches without the approval of Program Directors. While some obvious gaps exist in cost assignment for these services by these methods, a basis for further improvement has been established and it is planned to repeat this procedure again in FY 1986. Inequities in work distribution were uncovered by the study, and criteria for resource requirement estimation was provided. Resource requirement for projects and studies which use Animal Husbandry services can now be estimated.

A decision was made to open the E Module animal facilities for long term and special purpose studies during FY 1985; however, it appears unlikely that this target will be met because of the unexpected loss of two Animal Husbandry Supervisory personnel. Preexisting supervisor shortages compounded this problem and it has proven difficult to recruit qualified replacement personnel at current salary levels. Although one supervisor has been recruited, a serious shortage remains. Progress has been made in preparing E Module physically for occupancy; nevertheless, the facility cannot be opened until additional supervisors are recruited. Delays in several projects are inevitable.

During 1985 significant strides were made in improving animal health. As of June 1985, epidemic rodent virus diseases were completely eliminated from all but two animal rooms throughout the Institute. One virus, mouse hepatitis virus, persists in two rooms in Bldg. 15. Steps have been initiated to depopulate these rooms and eliminate the infection; but this process will probably carry over into FY 1986 before it is satisfactorily completed. Elimination of this threat to NIEHS rodent populations would place the Institute rodent research colonies in a somewhat unique but enviable position, offering an unusual opportunity for investigators to conduct studies without the often destructive interference caused by rodent viruses. The present status has been achieved through a systematic approach to animal health and quality assurance without restricting freedom of investigators to conduct science.

The Glassware/Media Service added a second shift in the glassware washing operation to meet the demand for clean glassware. This shift is staffed with temporary employees and is necessary because of the limited processing capacity of current washing equipment. A more efficient machine has been purchased and is schedule for installation in October 1985. It is proposed to conclude the second shift at that time. Media service has further strengthened its quality assurance program and presently provides formulation data and descriptive physical-chemical characteristics to investigators on each batch of media prepared. Records of these characteristics are maintained by the Media Unit Office as reference information. With the assistance of the Quality Assurance Laboratory, stringent standards for media quality assurance have been established and a program of record keeping, monitoring, and reporting has been instituted. This program has received guidance and recommendations by senior investigators in the Institute and has been exceptionally well received. The goal of the program is preemptive evaluation of physical-chemical and microbial quality of media provided to investigators, and an atmosphere of mutual participation in problem solving.

Major contributions of an administrative nature were made by the Quality Assurance Laboratory during the current year. These include: 1) Positive identification of the source of several rodent diseases which caused serious problems among experiment populations; 2) Application of health surveillance procedures to help track down the source of animals of uncertain origin; 3) Examined the colony health profile of five potential new animal suppliers and recommended the acceptance of two new suppliers; 4) Played a significant role in the colony health improvement program described above, especially through the sentinel animal program; 5) Established a stringent quality assurance program for animal drinking water; 6) Reevaluated and modified contract specifications for animal feed and bedding; 7) Instituted a quality assurance audit for the Media Unit. This program includes media audit, records audit, record keeping, and consultation with the Media Unit Supervisor.

Other major Branch administrative activities include: 1) Full technical participation in rodent bioassays (methyl isocyanate, methyl bromide), including animal care, animal evaluation, observation, data collection, record keeping, and dosing; 2) Quarterly presentation of a Workshop on Humane Care and Use of Laboratory Animals (required attendance for all investigators and technicians working with animals); 3) Implementation of a preliminary review procedure for all applications to use animals in research, including record keeping and consultation with investigators; 4) Expansion of technical assistance to investigators working with animals, including instruction, demonstration, performance,

surgery, microsurgery, etc. This program is viewed as an important long range investment to the Institute and humane care and use of animals; 5) Revision of the Syllabus on the Humane Care and Use of Animals; 6) Investigation and consultation with investigators on matters of unexplained morbidity and mortality among experimental animals.

Research Activities

Research activities in CMB are problem solving in type. One current project extends work on the mouse bioassay for detecting estrogenic activity (EA) in animal diets. Previous work defined parameters necessary to conduct EA bioassays using the CD-1 mouse. These parameters include age at weaning, duration of exposure to test diets, method of specimen collection, normal uterine growth curves, suitable age for exposure, and statistical evaluation of results. Results of these studies have been submitted for publication. These methods were applied to the EA bioassay of semipurified (AIN-76), certified, and standard rodent diets including breeder and maintenance diets. Using statistical methods, it was shown that AIN-76 modified to contain 4% corn oil and breeder diet (Purina 5015) both contained EA equal to or greater than 4-6 ppb DES. Two significant conclusions may be reached: 1) These studies demonstrate the sensitivity of properly conducted mouse EA bioassays for detecting DES and presumably other EA substances in rodent diets, and suggest that this assay may be useful as a screening test for EA activity in other diets including possibly human diets; 2) Some variability exists in EA activity in rodent diets used for research animals and this should be considered when certain types of studies involving estrogen receptor activity, organogenesis, reproductive toxicology, etc., are planned.

Further studies on the characteristics of rabbit coronavirus (RbCV) were conducted in collaboration with investigators from Johns Hopkins, NADL, and other units within NIEHS. RIA, plaque serum neutralization (PSN) and gel diffusion (GD) were used to test the relatedness of antisera to RbCV to other coronaviruses including transmissible gastroenteritis (TGE), canine coronavirus (CCV), feline infectious peritonitis (FIP), and calf diarrhea coronavirus (CDCV). TGE, CCV, and FIP cross reacted strongly in the RIA test; TGE and CCV cross reacted slightly in the PSN test; none cross reacted in the GD test. Two of three rabbits vaccinated with CCV survived challenge with RbCV, as did one of three vaccinated with FIP. TGE, CCV, and FIP all belong to the Group II (Pedersen) coronaviruses. Previous work demonstrated two-way cross reactivity to human coronavirus 229E, also a Group II coronavirus. Evidence thus suggests that RbCV more closely aligns antigenically with the Group II viruses, but more sophisticated tests are necessary to establish this. The myocardial tropism of RbCV has been previously described.

Studies are in progress to determine the relationship of saprophytic Mycobacterium chelonae to the observation of microgranulomas in the liver of rodent species used for experimentation and in bioassays. M. chelonae was isolated from deionized water fed to experimental rodent populations. When administered by various routes to experimental mice (i.p., i.n., gavage, drinking water), granulomas similar to naturally occurring lesions were observed in the liver. Lesions persisted for 35 days PI in euthymic mice and acid fast organisms were observed 50 days PI in the livers of athymic mice. Organisms were not recoverable at either of these time points, but were recoverable at 7 days PI and their presence within granulomas establishes their histopathological

relationship to the lesions. M. chelonae is ubiquitous in the environment and quickly colonizes water purification systems where careful management is necessary to eliminate it. The biological consequences of the microgranulomas from the experimental standpoint are uncertain. Dose response studies are now in progress.

Studies on the natural history of mouse hepatitis virus (MHV) continue a project held in abeyance during the past year. Transmission of MHV in "natural" settings has not been thoroughly understood in spite of our long acquaintance with this murine coronavirus. Euthymic sentinel mice placed in suspect animal rooms seroconverted on ELISA test in irregular patterns and rates, making interpretation uncertain in some instances. Athymic sentinels developed predictive, and finally classical lesions of hepatitis, thus establishing the sensitivity of this method of detection. These observations on the sensitivity of the athymic sentinel reconfirm observations of others. Further studies in "unnatural" settings, where naive cohabitants were placed with experimentally infected CD-1 mice for 3 day periods, then removed and isolated, showed that under these circumstances a narrow window of patency existed for virus shedding. Cohabitants as well as fecal slurry gavaged naive mice only expressed antibody response to MHV when exposed to experimentally infected mice during the period 12-18 days PI when examined by the ELISA method. These studies will be repeated to confirm this observation. Studies will also be conducted to determine if the aerosol method is important in the natural epidemiology of MHV.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 22102-04 CMB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization of a Coronavirus from Rabbits		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
P.I.:	J. D. Small	Head, Diagnostic Laboratory CMB, NIEHS
Others:	M. E. Clements	Bio. Lab. Tech. CMB, NIEHS
COOPERATING UNITS (if any) Chemical Pathology Branch, TRTP, NIEHS, NIH (Dr. M. Thompson); Division of Comparative Medicine, Johns Hopkins School of Medicine (Drs. J. Strandberg and L. Aurelian); National Animal Disease Center, ARS, USDA, Ames, IA (Dr. R. D. Woods)		
LAB/BRANCH Comparative Medicine Branch		
SECTION Diagnostic Laboratory		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS .3	PROFESSIONAL .2	OTHER .1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.) <p>The objective of this project is to study the pathogenesis of rabbit coronavirus (RbCV), the rabbit's physiologic response to this virus, and the relatedness of RbCV to other members of the Coronaviridae. RbCV antiserum was shown to cross react strongly with transmissible gastroenteritis virus (TGE), canine coronavirus (CCV), and feline infectious peritonitis (FIP) in the RIA, slightly to TGE and CCV, but not FIP in a plaque serum neutralization and not at all in a gel diffusion test. Two of 3 rabbits vaccinated with CCV and 1 of 3 vaccinated with FIP survived challenge with RbCV. None vaccinated with TGE or calf diarrhea coronavirus (CDCV) survived challenge. Single rabbits given RbCV reacted with FIP antiserum or TGE antiserum did not survive. Based on previous work showing a 2 way cross with coronavirus 229E and this new data, RbCV is most probably in Group II of the Coronaviridae [229E (Human), TGE (swine), FIP (cat), CCV (dog)]. Nucleotide homology studies will be required to prove this supposition. Such studies are hampered by the present inability to grow this virus in tissue culture. Two of 3 rabbits given RbCV reacted with CCV antiserum for 2 hours survived.</p> <p>The apparent protective effect of CCV vaccination will be explored further. Additional studies to understand the relatedness of RbCV to the other coronaviruses will be done. Assessment of myocardial damage using ECGs and creatine kinase isozymes are in progress. In cooperation with a team of virologists, further attempts will be made to adapt RbCV to tissue culture.</p> <p>The significance of this work lies in the ability to study a viral disease with a cardiotropism in an animal of sufficient but manageable size to allow sequential clinical and physiological observations. The damage to the rabbit heart by RbCV has a corollary in the human heart with the Coxsackie viruses, <u>Mycoplasma pneumoniae</u>, influenza virus, <u>Herpes zoster</u>, and possibly other infectious agents.</p>		

PROJECT DESCRIPTION

PERSONNEL ENGAGED: J. D. Small, DVM, MPH, Head, Diagnostic Laboratory, CMB, NIEHS; M. Thompson, DVM, Chemical Pathology Branch, NIEHS; J. Strandberg, DVM, PhD, and L. Aurelian, PhD, Division of Comparative Medicine, Johns Hopkins School of Medicine; R. D. Woods, PhD, National Animal Disease Center, ARS, USDA.

OBJECTIVES: The objective of this project is to study the pathogenesis of rabbit coronavirus (RbCV), the rabbit's physiologic response to this virus, and the relatedness of RbCV to other members of the Coronaviridae.

METHODS EMPLOYED: Inoculation of rabbits, collection of blood, and clinical chemistry assays were by previously reported methods. Antibodies to transmissible gastroenteritis virus (TGE, a porcine virus), calf diarrhea coronavirus (CDCV), feline infectious peritonitis virus (FIP), and canine coronavirus (CCV) were raised using commercial vaccines (TGE and CDCV) and/or virus grown in tissue culture in the laboratory (FIP, CCV, TGE). In vivo challenge studies were done using the rabbits in which antibody was raised. The stock of rabbit coronavirus was the same in each case. In vivo serum neutralization tests were done as previously published using rabbit anti-TGE, -FIP, and -CCV against rabbit coronavirus (RbCV). Rabbit raised antibodies to TGE, CDCV, and RbCV were measured and compared against TGE, CCV, and FIP in three systems (gel diffusion, plaque serum neutralization, and RIA).

MAJOR FINDINGS AND PROPOSED COURSE: RbCV antiserum cross reacted strongly with TGE, CCV, and FIP in the RIA, slightly to TGE and CCV but not FIP in a plaque serum neutralization and not at all in the gel diffusion test. Rabbits vaccinated against TGE and CDCV did not survive challenge. Two of 3 vaccinated with CCV and 1 of 3 vaccinated with FIP survived challenge. Antiserum to CCV but not TGE or FIP partially neutralized RbCV. Assessment of myocardial damage using ECGs and CK isozymes will begin shortly. Likewise, further examination of coagulation factors, activated partial thromboplastin time, prothrombin time, thrombin time and fibrinogen levels are planned. Serum from rabbits vaccinated with RbCV, TGE, CDCV, FIP, and CCV will be further examined for antibody titers. The apparent protective effect of CCV vaccination will be further explored. Through collaborative efforts, further attempts will be made to grow this virus in tissue culture.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The coronaviruses are a large group of species specific viruses which frequently share common antigens. They present a variety of clinical manifestations, due to enteric, respiratory, and neurologic tropisms. RbCV is the first coronavirus identified with a myocardial tropism. The significance of this lies in the ability to study a viral disease with a cardiotropism in an animal of sufficient but manageable size to allow sequential clinical and physiologic observations. The damage to the rabbit heart by RbCV has a corollary in the human heart with the Coxsackie viruses, Mycoplasma pneumoniae, influenza virus, Herpes zoster, and possibly other infectious agents.

The study of the Coronaviridae in the broader scope has value because in mice and rats these viruses (MHV and RCV/SDA) are responsible for the majority of morbidity in NIEHS animals as well as in animals of other institutions. This morbidity has been responsible for the loss of several projects in recent years resulting in a loss of time and money. A greater understanding of the biology of the coronaviruses and the development of vaccines to those of the mouse and rat would be most helpful.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 22103-02 CMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Natural History of Mouse Hepatitis Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: C. B. Richter Chief CMB, NIEHS

Others: J. E. Thigpen Head, Quality Assurance Lab CMB, NIEHS
E. H. Lebetkin Biol. Lab. Tech., QAL CMB, NIEHS

COOPERATING UNITS (if any)

Chemical Pathology Branch, Toxicology Research & Testing Program (F. A. Talley)

LAB/BRANCH

Comparative Medicine Branch

SECTION

Office of Chief, Quality Assurance Lab

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

.75

PROFESSIONAL

.5

OTHER

.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

Transmission of mouse hepatitis virus (MHV) was studied in a "natural" animal room setting using euthymic and athymic mice; and in unnatural settings where experimentally infected cage mates served as time-controlled donors.

"Natural" setting. Euthymic sentinel CD-1 mice placed in nonfilter capped cages in animal rooms suspected of harboring endemic MHV were examined at intervals for seroconversion to MHV by the ELISA method. First results of serological tests produced a mixture of non converters and low reading converters, making interpretation difficult or uncertain. Athymic sentinels killed at the same time points and examined for evidence of MHV infection had histological criteria that predicted insipient characteristic lesions at 7 and 11 weeks post placement. At 13 and 17 weeks athymic mice had fully developed MHV lesions. The length of time necessary for lesions to develop in athymic mice is difficult to predict but the sensitivity of system appears to be of a high order.

Unnatural setting. CD-1 weanling mice were experimentally infected with MHV by the i.p. and oral routes. Beginning at 3 days PI these mice were placed in sterile cages and cohabited with uninoculated CD-1 weanlings known to be free of MHV infection. Cohabitants were removed after three days and replaced by new virus free weanlings. This procedure was repeated with seven groups of cohabitants. The last group of cohabitants was allowed to remain with the infected mice for 7 days (21-28 days PI). Upon removal from infected mice, each group of cohabitants was isolated in a Horsfall unit for 28 days, then bled for serological examination. A second cohort of virus free weanlings was inoculated orally with a slurry of feces collected each time cohabitants were removed from infected mice. Only the virus free weanlings exposed to infected mice between 12-15 and 15-18 days PI and mice inoculated with slurry from these same time points seroconverted positive for MHV. No other groups or slurry cohorts seroconverted.

PROJECT DESCRIPTION

PERSONNEL ENGAGED: C. B. Richter, Chief, Comparative Medicine Branch;
J. E. Thigpen, Head, Quality Assurance Laboratory; IRP, NIEHS.

OBJECTIVES: To study the natural history and transmission of mouse hepatitis virus (MHV) in the animal facility setting. To gain insight into methods of control of the infection.

METHODS EMPLOYED: One of us (Thigpen) has previously investigated MHV infection by: 1) tracking "natural" seroconversion of sentinel mice placed in animal rooms known to contain infected mice, and in rooms of uncertain status; 2) demonstrating that cohabitation of naive euthymic and athymic mice with suspected infected mice is a useful test for identifying infected mice; 3) demonstrating that feces and bedding from cages of infected mice contain enough virus to confer infection upon naive mice placed in contact with these materials. In the studies reported here: 1) Sentinel athymic and euthymic were placed in mouse rooms considered to contain endemic MHV infection and killed at 5, 7, 11, 13, 17 weeks and examined histologically and serologically for evidence of MHV infection; 2) Naive mice were placed with experimentally MHV infected CD-1 weanling mice for 3 day periods at 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, and one long period, 21-28 days PI. Each group of naive cohabitants was then isolated in a Horsfall unit for 28 days and tested for serological evidence of MHV by the ELISA method. As a further test of virus shedding, feces collected from infected cages at each cohabitant removal time were mixed into a slurry and used to gavage naive CD-1 weanlings. These were also isolated in Horsfall units and examined serologically at 28 days PI.

MAJOR FINDINGS: Euthymic sentinel animals seroconverted positive for MHV at variable rates and in irregular patterns. Some sentinels seroconverted as early as one month, some took as long as 18 weeks. Athymic sentinels became histologically suspicious as early as 7 weeks, and histologically and macroscopically positive as early as 13 weeks and as late as 17 weeks post introduction. Naive mice cohabited with experimentally infected mice and naive mice gavaged with fecal slurry from experimentally infected mice, both seroconverted positive only if they were exposed by contact, or by slurry, during the periods 12-15 and 15-18 days after the experimental infections.

SIGNIFICANCE: 1) Many variables within the animal room are likely to influence the rate of spread of MHV. Thus, lengthy periods may elapse before proof of infection can be obtained. Nevertheless, sentinel animals are sensitive indicators and the athymic mouse is especially useful in uncertain circumstances. 2) Infected mice may only have a narrow window of virus shedding. If this is true, specific control measures might be influenced by this observation.

PROPOSED COURSE: Experiments which define the patent period of MHV shedding will be repeated and expanded. Experiments to demonstrate that cage-to-cage spread is or is not via the aerosol route will be designed; and IFA studies to identify the earliest period of infection detection will be started during the next calendar year.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 22104-02 CMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Assessment of the Mouse Bioassay Test for Detecting Estrogenic Activity in Feed

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	J. E. Thigpen	Microbiologist	CMB, NIEHS
Others:	E. H. Lebetkin	Bio. Lab. Tech.	CMB, NIEHS
	L. A. Li	Biostatistician	BRAP, NIEHS
	J. K. Haseman	Biostatistician	BRAP, NIEHS
	C. W. Jameson	Chemist	TRTP, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Comparative Medicine Branch

SECTION

Quality Assurance Laboratory

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

.8

PROFESSIONAL

.3

OTHER

.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously described the necessary parameters for conducting the mouse bioassay for detecting estrogenic activity in animal diets, including a standardized method for removing, trimming, and weighing the uterus, age and strain of test pups, and methods of statistical evaluation. Statistical evaluation (t-test) of bioassay data has shown that uterine weight alone, without calculated uterine:body weight ratio, is adequate to determine a statistically significant (P.05) difference between control diets containing 0, 4, or 6 ppb DES. We have also employed the mouse bioassay using the CD-1 mouse to compare the estrogenic activity of semipurified, certified, standard and open formula rodent diets. Mice fed semipurified diet (AIN-76), modified semipurified diet containing 4% corn oil, or a natural ingredient breeder diet (Purina #5015), demonstrated significant (P less than .05) increases in uterine weight at 5, 7, and 9 days post weaning, when compared to other natural ingredient diets. This level of estrogenic activity was equal to or greater than the activity observed in a natural ingredient maintenance diet (Purina #5002), containing 4 or 6 ppb of DES.

These results suggest: 1) significant differences exist in the level of estrogenic activity in some commercially available diets; 2) the importance of the diet when performing or comparing fertility, reproduction, organogenesis, toxicological or estrogenic studies; 3) a standardized diet with minimal estrogenic activity may be desirable for some studies.

PROJECT DESCRIPTION

PERSONNEL ENGAGED: J. E. Thigpen, PhD, Quality Assurance Lab, CMB, NIEHS; L. A. Li, PhD, and J. K. Haseman, PhD, Biometry and Risk Assessment Program, NIEHS; C. W. Jameson, PhD, Toxicology & Research Testing Program, NIEHS

OBJECTIVES: The objective of this project was to compare the estrogenic activity of semipurified (AIN-76), modified semipurified, certified (Purina #5002), standard (Purina #5001 and #5015) and open formula (NIH-07 and NIH-31) rodent diets using the mouse bioassay method.

METHODS EMPLOYED: Female pups from CD-1 dams with standardized litters were weaned at 15 days of age and randomly assigned to test diets containing 0, 4 or 6 ppb added diethylstilbestrol (DES). Mice were housed 5 per cage and given deionized water and feed ad libitum. Uterine and body weights were recorded on 15 mice per diet at 3, 5, 7 and 9 days post weaning.

MAJOR FINDINGS AND PROPOSED COURSE: Mice fed semipurified diet (AIN-76), modified semipurified diet containing 4% corn oil, or a natural ingredient breeder diet (Purina #5015), demonstrated significant (P less than .05) increases in uterine weight at 5, 7 and 9 days post weaning, when compared to other natural ingredient diets. This level of estrogenic activity was equal to or greater than the activity observed in a natural ingredient maintenance diet (Purina #5002), containing 4 or 6 ppb of DES. These results suggest significant differences exist in the level of estrogenic activity in some commercially available diets; 2) the importance of the diet when performing or comparing fertility, reproduction, organogenesis, toxicological or estrogenic studies; 3) a standardized diet with minimal estrogenic activity may be desirable for some studies. Presently, we plan to summarize the data and prepare manuscripts for publication before embarking on any additional bioassay studies.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A major source of exogenous estrogenic substances, which may affect laboratory animals, comes from their diet. Naturally occurring plant products, pesticides, feed additives, and some related steroids may exhibit estrogenic activity when consumed by laboratory animals. Research at NIEHS includes extensive studies in reproductive and developmental toxicology; hence, dietary constituents that act as modifiers of the female mouse genital tract physiological state are undesirable. The wide range of potentially active estrogenic substances renders specific analysis impractical and uneconomical. The mouse bioassay remains the single most useful test to detect estrogenic activity in feeds and foodstuffs, but must be carefully controlled. The bioassay is applicable to human diets and, therefore, may be useful in investigating the source(s) of unexplained estrogenic activity/substance(s) causing premature sexual development in female children among certain ethnic populations and geographical locations.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 22106-01 CMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Characterization of an Acid Fast Bacterium Isolated from Animal Drinking Water

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: J. D. Small Head, Diagnostic Laboratory CMB, NIEHS

Others: M. E. Clements Bio. Lab. Tech. CMB, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Comparative Medicine Branch

SECTION

Diagnostic Laboratory

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

.5

PROFESSIONAL

.3

OTHER

.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The objective of this project is to characterize and study the pathogenic potential of an acid fast (AF) bacterium repeatedly isolated from an ultrapure water source furnishing drinking water to some of the Institute's animals. The organism has been identified as Mycobacterium chelonae. Inoculation of euthymic and athymic mice by IV, and euthymic mice by IP, IN, and gavage routes and in the drinking water produced lesions in the liver and, by the IN route, lesions in the lungs. No deaths were observed, even when mice were given cortisone acetate. Mycobacteria were recovered from livers of euthymic mice inoculated 7 days earlier but not thereafter. Granulomas were observed in the liver from post inoculation day (PID) 2 until day 35 when the experiment was terminated. The lesion was greatest in size and reaction in mice killed on PID 10 and 14. By day 35 lesions were well defined resolving granulomas; but, AF bacteria were rare. Acid fast bacteria were observed in livers of athymic mice on PID 50; however, no bacteria were recovered by culture and lesions were minimal or absent.

The number of bacteria required to produce lesions will be examined as will ways to increase the likelihood of recovering the organism from the liver. Spontaneous liver lesions will be searched for AF organisms. Isolator maintained mice and rats will be compared to room maintained animals. Other water borne organisms will be examined for their ability to induce comparable liver lesions.

This work is significant because of the many similar focal lesions of undetermined origin observed in livers of animals on chronic bioassays conducted under the NTP. Identifying waterborne bacteria and a Mycobacterium sp. in particular as the cause would allow for corrections in management practices.

PROJECT DESCRIPTION

PERSONNEL ENGAGED: J. D. Small, DVM, MPH, Head, Diagnostic Laboratory, CMB, NIEHS

OBJECTIVE: The objective of this project is to characterize and study the pathogenic potential of an acid fast bacterium repeatedly isolated from an ultrapure water source furnishing drinking water to some of the Institute's animals.

METHODS EMPLOYED: Mycobacterium chelonae isolated from the deionized water line supplying the Bldg. 101 C&D Module bottle filler was grown in broth for 72 hrs, centrifuged, washed 2X with saline, resuspended, and inoculated by various routes into mice (B6C3F1, C57BL/6, CD-1 and nu/nu) or added to their drinking water. Dilutions of the bacterial suspension were counted on spread plates of standard plate count agar (TGYF). Mice were killed from 2 to 50 days following inoculation or feeding and livers were divided for histology and culture. Liver for culture was ground in 2 ml of saline and inoculated into TSB and, in some cases, TSB+penicillin and Lactobacillus Selection Broth (LBS). After incubation at 35°C for 48 hrs TSB and TSB+penicillin were streaked on TGYA and LBS broth on LBS agar.

MAJOR FINDINGS AND PROPOSED COURSE: My. chelonae induced liver lesions by all routes used (IV, IP, IN, GAVAGE and Drinking Water). The IN route induced low grade pulmonary lesions as well. The IV route induced the most severe liver lesions. No mice died following inoculation. My. chelonae was not recovered from the liver following inoculation except in mice challenged IV, and then only during the first week. Liver lesions were most severe in nu/nu mice killed on day 14. Athymic mice killed on day 50 had rare microfoci of necrosis; however, careful examination of liver sections revealed acid fast bacteria. Isolator maintained animals given sterile water will be compared to room maintained animals given deionized water. The number of bacteria required to induce the observed lesion will be examined as will ways to enhance recovery of the organism. Other bacteria isolated from the water supply will be examined for their ability to induce this lesion.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This work is significant because of the numerous similar focal lesions of undetermined origin observed in livers of mice and rats on bioassay studies conducted under the NTP. Identifying water borne bacteria and a Mycobacterium sp. in particular as the cause would allow for corrections in management practices.

TOXICOLOGY RESEARCH AND TESTING PROGRAM

TOXICOLOGY RESEARCH AND TESTING PROGRAM

Summary Statement

The Toxicology Research and Testing Program (TRTP), the National Institute of Environmental Health Sciences (NIEHS) component of the National Toxicology Program (NTP), develops scientific information about potentially toxic and hazardous chemicals. [The two other components of the NTP are the National Center for Toxicologic Research (FDA) and the National Institute for Occupational Safety and Health (CDC)]. This toxicology information is used for protecting the health of the American people and for the primary prevention of chemically-induced diseases. TRTP concentrates activities in toxicology research, assessment of toxic potential of chemicals, development/validation/evaluation of toxicology methods and assays, and provides toxicological information to the Government research and regulatory agencies and to the scientific and public communities. Four specific and continuing aims center on:

- Expanding toxicological profiles of the chemicals nominated, selected, and studied.
- Increasing as necessary, and as funds permit, the number and rate of chemicals studied and evaluated toxicologically.
- Developing and validating a series of experimental designs, protocols, and biologic assays appropriate for research and regulatory needs.
- Using a coordinated communications network to collect, evaluate, share, and disseminate toxicological information.

To accomplish these major goals, the program segments are grouped into discipline-oriented yet fully coordinated branches: Carcinogenesis and Toxicologic Evaluation (Dr. D. Bristol), Cellular and Genetic Toxicology (Dr. R. Tennant), Chemical Pathology (Dr. G. Boorman), Systemic Toxicology (Dr. B. Schwetz), and Data Management and Analysis (Dr. D. Hoel; this branch is part of the Biometry and Risk Assessment Program). These functions include scientific and technical monitoring of collaborating laboratories and oversight of financial data bases that relate to toxicology activities. Each of these discipline areas and their accomplishments are described separately in the sections that follow this overview.

Individual NTP scientists serve as the center for a particular program activity and are responsible for developing (in collaboration with other NTP colleagues) the subprogram objectives and the implementation plans, as well as the coordination and supervision of the program work. Further, the program leaders are responsible for the development and supervision of contracts that extend these activities or that perform in-depth toxicologic characterization of chemicals.

The strategy for assay and protocol development and validation examines existing and emerging methodologies to identify those that may be adequately sensitive and reproducible. Those found to offer improvement over older methods are selected for further investigation and perhaps validation. When basic research findings suggest new areas of toxicology studies, TRTP will undertake the appropriate methods development and validation.

- In methods development/validation, emphasis is given to in vivo mammalian and human somatic cell assay development including: (1) mouse

bone marrow cytogenetics; (2) evaluation of the mouse erythrocyte micronucleus assay; and (3) protocol development and data collection assessing frequencies of chromosome aberrations and sister chromatid exchanges in human lymphocytes. Developmental work was completed for in situ monitoring of mutagens in the workplace. Validation of assay methodology for measuring germ cell mutations continues as well as cross-assay comparisons among the morphological specific locus, biochemical specific locus, and heritable translocation assays. Efforts continue to refine short-term rat liver tumor models for evaluating and interpreting the initiation/promotion mechanisms of chemicals known to be hepatocarcinogenic in rodents.

- Major emphasis remains on designing broadened yet specifically tailored protocols for each chemical in the prechronic phases (usually toxicology studies of six months or less in duration) to include a battery of genetic toxicity assays, chemical disposition studies, measures of alterations in reproduction and fertility, and other target organ effects as well as clinical and morphological pathology. Studies in biochemical toxicology continue to be concerned with characterizing monooxygenase enzymes which metabolize chemicals, often to activated or toxic products. New initiatives were begun to characterize the toxicology of 1,3-butadiene and the structurally-related chloroprene and isoprene. Studies showing the first clear evidence of carcinogenicity of benzene in rodents have been completed and announced; these findings led to further studies underway on the metabolism and carcinogenic mechanisms of benzene.
- Ongoing initiatives include studies to explore the possible relationship of dietary oil or oils used as vehicles for oral studies and pancreatic cancer in male rats. Studies are being designed to assess the influence of various levels of dietary restriction on the occurrence of non-neoplastic (toxic) and neoplastic (carcinogenic) lesions in control animals. Continuing were short-term and long-term studies on the toxicology and potential carcinogenicity of chemicals and substances in foods, the environment, and the workplace.
- Microencapsulation is a new process in which a thin and continuous polymeric coating is applied to particulate solids or liquid droplets as a means of administering reactive, volatile, or unpalatable chemicals in the feed, rather than by the gavage route. 2,6-Xylidene and trichloroethylene (TCE) were encapsulated in gelatin-sorbital by the centrifugal extrusion process. These capsules were stable in rodent feed. Bioequivalence in rats was shown for microcapsulated TCE and neat chemical. In fourteen-day studies, there was no significant loss of TCE from the feed, and similar toxic effects were seen in animals receiving the microencapsulated TCE (by feed) and those given neat TCE in corn oil (by gavage). These bioavailability and toxicology studies demonstrate that microencapsulation provides an appropriate and adequate method for studying the toxicological properties of many chemicals by the oral route. Longer term studies are in progress.
- Studies on survival and age-associated diseases and neoplasia have been completed and published on life-time studies of the Fischer 344/N rat; similar studies are underway using the B6C3F₁ mouse to determine mean

lifespan, age related changes in hematology and clinical chemistry, morphological changes in tissues exhibiting age related diseases, as well as age associated types and incidences of neoplasia. Like studies are being designed to evaluate the hamster as another species for use in toxicology and carcinogenesis studies.

- Oncogene activation has been observed in rodent neoplasms induced by chemicals. Oncogene activation has been investigated (TRTP and BRAP) by DNA transfection techniques in neoplasia taken from the B6C3F₁ mouse and Fischer 344/N rat. A marked difference in the presence of activated oncogenes was observed in rat tumors from untreated rats versus those from mouse liver tumors. All rat tumors examined did not yield activated oncogenes (0/29), whereas 30% (3/10) of mouse hepatocellular adenomas and 77% (10/13) of hepatocellular carcinomas scored positive by DNA transfection. These transforming genes were identified as an activated H-ras gene in all (3/3) the adenoma transfectants and in 8 of the 10 carcinoma transfectants. The two remaining hepatocellular carcinomas contained transforming genes which do not appear to be members of the known ras gene family. The B6C3F₁ mouse liver system might provide a sensitive assay for assessing not only the potential of a chemical to activate a cellular proto-oncogene, but also to detect various classes of proto-oncogenes which are susceptible to mutational activation.
- Nuclear magnetic resonance (NMR) imaging is a noninvasive technique that produces high resolution images in internal organs and structures. The technique depends upon the inherent property of atomic nuclei that have charge and net spin to generate small magnetic fields and thereby being susceptible to alignment in a strong external magnetic field. In addition to normal anatomic detail, various pathologic processes can be detected with NMR imaging. These include ischemia and infarction, abscessation, hematoma formation, degenerative diseases, malformations, and neoplasia. The ability to detect neoplasms and other lesions using NMR imaging are areas of active collaborative research interests of TRTP, IRP, and Duke University. Within the last nine months, significant progress has been made in the refinement of software and hardware for imaging small laboratory animals (rats). Multiple, discrete hepatic neoplasms ranging from 2 to 12 mm in diameter were readily identified. The images were correlated with gross and histologic sections of the animals. Preliminary imaging experiments have been conducted on rats with mononuclear cell leukemia and to demonstrate normal anatomic structures in the brain. Imaging is ongoing to detect pituitary neoplasms in rats. Future studies will examine sequential changes in the livers of rats exposed to hepatic carcinogens to examine time to tumor formation, the biologic behavior, and characteristics of hepatic neoplasms and the regression/progression of neoplasms.
- Publications: during the last 15 months, TRTP staff members published 51 journal articles, 22 books or book chapters, and presented 65 abstracts at national meetings.

A major effort on post study, Good Laboratory Practice/Quality Assurance audits of experimental data has been implemented. Four additional audit teams have been added which enable the TRTP to conduct more intensive GLP and in-life data

audits. All toxicology and carcinogenesis studies receive an indepth examination of the chemistry, toxicology, and pathology data and informational records before TRTP/NIEHS draft technical reports are presented to the NTP Board of Scientific Counselors Peer Review Panel for evaluational approval. All studies except those from a single laboratory have been shown during data audits to support the data collected and evaluated in Draft Technical Reports.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-ES-21064-03-TRTP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Bioavailability of TCDD in Missouri Soil

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E.E. McConnell Acting Director TRTP NIEHS

OTHERS: M.W. Harris Biological Laboratory Technician TRTP NIEHS

J.D. Allen Biological Laboratory Technician TRTP NIEHS

E. Haskins Biological Laboratory Technician TRTP NIEHS

COOPERATING UNITS (if any)

Environmental Protection Agency
Laboratory of Molecular Biophysics, NIEHS

LAB/BRANCH

Office of the Director for Toxicology Research and Testing Program

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0.4

PROFESSIONAL:

0.2

OTHER

0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

TCDD (Dioxin) contaminated soil from two sites in Missouri is being investigated to determine the bioavailability in soil. Guinea pigs are being used in the investigation. Results suggest that there is high bioavailability of TCDD in dirt after ingestion. Studies on bioavailability via the skin are in progress in a second species.

PROJECT DESCRIPTION

METHODS EMPLOYED: Hartley strain guinea pigs will be given various amounts of contaminated soil via gavage. TCDD spiked soil as well as TCDD in corn oil will be used as positive controls. Parameters being studied include clinical signs and histopathology.

MAJOR FINDINGS AND PROPOSED COURSE: Study was started in June 1983.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Relatively large amounts (600-800 ppb) of 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) have been found in soil from two different locations in Missouri. It is extremely important to establish the bioavailability of TCDD in this soil to accurately determine the hazard to humans exposed to such an environment.

PUBLICATIONS

McConnell, E.E., Harris, M.W., Harvan, D.J., Albro, D.W. and Hass, J.R.: Studies on the bioavailability in Guinea Pigs of Dioxin in Soil. Society of Toxicology meeting, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01-ES-21066-03 TRTP
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Studies on the Etiology of the Spanish Toxic Oil Syndrome		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: E.E. McConnell OTHERS: M.W. Harris J.D. Allen	Acting Director Biological Laboratory Technician Biological Laboratory Technician	TRTP TRTP TRTP NIEHS NIEHS NIEHS
COOPERATING UNITS (if any) The Government of Spain WHO Regional Office for Europe, Copenhagen, Denmark		
LAB/BRANCH Office of the Director for Toxicology Research and Testing Program		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 0.1	PROFESSIONAL 0.0	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided) Samples of suspect <u>olive/rapeseed</u> oil which were suspected of being part of the <u>Spanish Toxic Oil Syndrome</u> were given to <u>guinea pigs</u> and <u>ducklings</u> . The theory being tested was to rule out the presence of an obscure mycotoxin such as <u>cytochalasin</u> or <u>trichothecene</u> . The suspect oil samples did not produce disease in those laboratory species. Studies finished.		

PROJECT DESCRIPTION

METHODS EMPLOYED: Seven suspect oil samples were given by gavage to weanling guinea pigs and ducklings (1 week of age) by subcutaneous injection. Controls received pure olive oil or rapeseed oil. The animals were observed for 14 (ducks) or 30 (guinea pigs) days at which point they were killed and subjected to histopathologic examination. An additional study using mice will be conducted using a different suspect oil sample. This study is being coordinated by WHO and will be conducted using the same oil and protocol in Spain, the United Kingdom (MRC Laboratories) and the U.S. (NIEHS). Results from the three laboratories will be compared.

MAJOR FINDINGS AND PROPOSED COURSE: No adverse clinical signs or pathologic effects were observed with the first oil samples. It is suspected that these samples of oil were not part of the Spanish Toxic Oil Syndrome.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: To date the etiologic factor responsible for sickness of >30,000 and death of >1000 Spaniards has not been determined. There is definite epidemiologic data which incriminates adulterated olive oil (with rapeseed oil, etc.) as the cause of the illness, but the specific etiologic factor in the oil has not been identified. The disease syndrome is felt to be related to endothelial damage. It would be extremely important to identify the etiologic factor so that such a disaster might be prevented in the future.

CARLTECH ASSOCIATES, INC. - Rockville, Maryland 20852
(N01-ES-3-5020)

TITLE: Bioassay Report Preparation

CONTRACTOR'S PROJECT DIRECTOR: William D. Theriault, Ph.D.

PROJECT OFFICER (NIEHS): J. E. Huff, Ph.D., Assistant to the Director

DATE CONTRACT INITIATED: September 30, 1982

CURRENT ANNUAL LEVEL: \$552,122

PROJECT DESCRIPTION

OBJECTIVES: The contractor provides editorial, word processing, typesetting, statistical, and general support in the preparation of the National Toxicology Program's series of Technical Reports on toxicology and carcinogenesis studies of selected chemicals.

METHODS EMPLOYED: From records and data provided by contract testing Laboratories the report preparation support contractor prepares summaries of the experimental protocols and results of NTP studies of the effects of selected chemicals on rodents. NTP scientists evaluate the findings and prepare overviews of the chemicals and interpretative discussions of the results of the studies; the report preparation support contractor then prepares draft Technical Reports based on NTP's revisions for public review and then prepares camera-ready drafts for publication by the GPO. The report preparation contractor uses literature provided by the NTP, statistical programs, word processing equipment, and typesetting and other office equipment.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The Technical Report series is the public record of the NTP Toxicology and carcinogenesis studies and represents NTP's conclusions regarding the biologic effects of selected chemicals. These reports are often the only available data on the carcinogenic effects in animals of particular chemicals. The Technical Reports are distributed world-wide and oftentimes serve as the basis for decisions by other government agencies or by foreign governments.

IMMUQUEST LABORATORIES, INC. - Rockville, Maryland 20850
(N01-ES-38047)

TITLE: Contract for Good Laboratory Practices (GLP) Monitoring Support Resource

CONTRACTOR'S PROJECT DIRECTOR: Pamela H. Errico, Ph.D.

PROJECT OFFICER (NIEHS): Carrie E. Whitmire, Ph.D.
Quality Assurance and Good Laboratory Practices
Toxicology Research & Testing Program

DATE CONTRACT INITIATED: October 1, 1983

CURRENT ANNUAL LEVEL: \$293,000

PROJECT DESCRIPTION

OBJECTIVES: The overall aim of this project is to provide NTP with support staff and resources to ensure that GLP compliance exists and is continually enforced in an efficient, effective manner by all contract laboratories that perform studies for the National Toxicology Program.

METHODS EMPLOYED: Methods employed include annual QA/GLP site visits to contract laboratories, GLP compliance assistance to new laboratories and in-depth data and report audits.

MAJOR FINDINGS AND PROPOSED COURSE: Although GLP compliance has been a requirement of the NTP for its contract laboratories since 1980, differences of interpretation and difficulties in implementation by those laboratories continue.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Since the NTP is fully committed to assuring that in vivo and in vitro toxicology and carcinogenicity studies are performed in a technically, scientifically and administratively credible manner, this monitoring support resource contract enhances NTP's position by maintaining their posture in these efforts.

DYNAMAC CORPORATION - Rockville, Maryland 20852
(N01-ES-38048)

TITLE: Good Laboratory Practices (GLP) Compliance Monitoring Support Resource

CONTRACTOR'S PROJECT DIRECTOR: Finis Cavendee, Ph.D.

PROJECT OFFICER (NIEHS): Carrie Whitmire, Ph.D.
Quality Assurance and Good Laboratory Practices
Toxicology Research & Testing Program

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$227,000

PROJECT DESCRIPTION

OBJECTIVES: To assist NTP in the following six functions: 1) assure contract laboratory compliance with GLP guidelines, 2) review completed chronic toxicity studies for accuracy and integrity, 3) assist new laboratories in establishing compliance programs, 4) review quality assurance procedures at contract laboratories not covered by GLP requirements, 5) develop procedures for auditing on-line computer collected data, and 6) coordinate workshops on GLP activities.

METHODS EMPLOYED: The methods to be used in attaining these objectives include the audit and validation of archival data, the onsite inspection of laboratory facilities and review of data and quality control procedures, the development of written materials on auditing and GLP compliance, and the writing of reports summarizing completed work.

MAJOR FINDINGS AND PROPOSED COURSE: Audits have been completed on the chronic studies of 19 chemicals (Function 2). Onsite inspections of laboratory facilities and review of data have been completed at two laboratories (Function 1).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:
Adherence to GLP requirements will increase the quality of work performed under contract to NTP and strengthen the toxicologic database. Technical reports prepared with the assistance of an audit will provide more useful and reliable information.

ARGUS RESEARCH LABORATORIES, INC. - Horsham, Pennsylvania 19044
(N01-ES-38049)

TITLE: Good Laboratory Practices Compliance Monitoring Support Resource Contract

CONTRACTOR'S PROJECT DIRECTOR: Jane E. Goeke, Ph.D.

PROJECT OFFICER (NIEHS): Carrie E. Whitmire, Ph.D.
Quality Assurance and Good Laboratory Practices
Toxicology Research & Testing Program

DATE CONTRACT INITIATED: October 1, 1983

CURRENT ANNUAL LEVEL: \$251,000

PROJECT DESCRIPTION

OBJECTIVES: To assure the integrity and quality of the data generated by the NTP testing contractors.

METHODS EMPLOYED: Auditing and inspection of testing facilities is carried out by a team of qualified personnel consisting of two toxicologists, three Quality Assurance Auditors, two histotechnicians and a pathologist. GLP compliance of present testing operations is determined by site visits to assess the effectiveness of the testing facilities' Quality Assurance Unit. The accuracy of the Technical Reports is determined by auditing the raw data and validation of the contents of the Draft Technical Reports.

MAJOR FINDINGS AND PROPOSED COURSE: To date, ten site visits have been carried out. Audits for 24 compounds have been initiated. A total of nineteen reports have been accepted with no further changes, and seven reports have been submitted for review or are being revised.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The audit and inspections provide a means of assuring that the contract testing facilities adhere to GLPs, that the NTP Technical Reports represent the raw data generated for that particular study and that the conclusions of the report are substantiated by the raw data.

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37830
(1Y01-ES-4-0128)

TITLE: Environmental Teratology Information Center

CONTRACTOR'S PRINCIPAL INVESTIGATOR: John Wassom

PROJECT OFFICER (NIEHS): Florence Jordan

DATE INTERAGENCY INITIATED: FY 1975

CURRENT ANNUAL LEVEL: \$385,000

PROJECT DESCRIPTION

OBJECTIVES: Chemical Teratogenesis Literature -- Developed and supported by the NTP since 1975, the Environmental Teratology Information Center collects, organizes, and disseminates information on chemicals tested for teratogenicity. The ETIC data file contains 37,099 (as of July 1984) records, the majority of which are available online from TOXLINE AND RECON. These 37,099 references identify 9700 chemical, physical or biological agents that have been tested. These include 7700 chemical agents containing 6907 unique chemicals identified with Chemical Abstract Service (CAS) Registry Numbers as well as the physical (heat, stress, etc.) and biological (age, diseases, etc.) agents.

METHODS EMPLOYED: ETIC, Located at NIEHS, has established a microform document library containing copies of 25,999/33,499 (78%) papers referenced in the computer file. Each citation has been indexed with all bibliographic information, common and taxonomic name of test object, and Chemical Abstract Service (CAS) Registry Number. Titles and abstracts may be searched using key words. Chemicals in the ETIC Agent Registry may be searched by primary name, synonym, CAS number, fragment as a part of a chemical name, and molecular formula.

CARCINOGENESIS AND TOXICOLOGY EVALUATION BRANCH

CARCINOGENESIS AND TOXICOLOGY EVALUATION BRANCH

Summary Statement

The Carcinogenesis and Toxicology Evaluation Branch (CTEB) of the NIEHS conducts applied research studies designed to detect and characterize the toxic potentials of chemical agents. Although most of these studies are conducted in rodent species or other experimental model systems, the results are relevant to and a major factor in estimating the toxic potentials of chemicals to humans. In addition to conducting chemical toxicity and carcinogenicity studies, the CTEB collaborates with other scientific staff at the Institute in developing and validating alternatives to standard toxicity tests, and conducts applied biological research in chemical toxicity.

The major effort of the CTEB staff during FY 1985 was the design, conduct, monitoring, evaluation, and reporting of toxicity studies completed off-site by contractual mechanisms. These studies encompass both prechronic (acute, short-term repeated-exposure, subchronic) and chronic (up to lifetime exposure) whole-animal tests employing morphological, clinical, functional and biochemical endpoints. Although many of these studies follow a standard protocol with regards to frequency of exposure and range of endpoints, each individual protocol is carefully tailored to the properties of the test chemical and the needs of the requesting agency.

The Collaborative Resources Group of the CTEB provides the essential aspects for the toxicology and carcinogenesis studies conducted by the National Toxicology Program, namely Analytical Chemistry, Chemical Health and Safety, and Collaborative Services. Group staff procure, analyze and monitor chemicals for these studies. The Chemical Health and Safety office monitors each testing laboratory and each study within a test facility for those factors which may adversely affect the proper research and testing environment. Each resource is provided by substantial in-house effort and supplemented by resource contracts.

In addition to chemical testing, the CTEB staff conduct a number of on-site and off-site research projects. Some of these involve collaborations with other NTP or NIEHS staff. All of these research projects are of direct relevance to, and conducted in support of, the goals of the NTP in providing sound, informative toxicologic data.

Extramural Research Highlights: Extramural research activities are conducted via contract mechanisms.

- The toxicity and carcinogenicity testing of approximately 250 chemicals is being performed under the NTP Master Agreement. This number includes chemicals in any of the stages from design through report preparation (CTEB staff).
- For the general in vivo toxicology studies, 86 chemicals were procured or synthesized and completely analyzed. In addition, 31 chemicals were obtained and analyzed for other Programs within NTP such as teratology studies, immunotoxicology studies, reproductive toxicology studies, continuous breeding experiments and in-house TRTP studies. Services were provided for the analysis of bulk chemical, chemical in test vehicles, methods development for quality assurance, including purity, stability (both bulk chemical

and chemical/vehicle mixtures) and concentration determinations, chemical residue analysis of body tissues and fluids and special handling for residual and reproducible chemicals. In addition, tissue and body fluid residue analyses were developed and performed to enhance data from toxicity experiments of 3 chemicals. Support was also provided to the Cellular and Genetic Toxicology Branch (CGTB) by analyzing 248 chemicals for purity and identity. The Analytical Chemistry Group also provides staff support for the data auditing activities of the TRTP. This included the review of 65 completed studies to assure the chemistry performed in support of these studies was adequate and accurately reported. The chemistry group maintains an in-house chemistry laboratory for the TRTP which provides routine support and collaborative research with several TRTP staff members (Dr. Jameson).

- The chemical health and safety aspects of the NTP are also the responsibility of the Collaborative Resources Group. Frequently, special safety requirements are needed because of a particular chemical's properties or the unique needs of the specific toxicology experiment. Input from chemical health and safety on NTP studies was provided for initial experimental design to insure special safety requirements are met and to provide chemical specific health and safety guidelines. Involvement continues through initial laboratory evaluation, followup site visits, program reviews, report monitoring, recommended changes in procedures, facilities design, etc., as well as response to problem emergency situations and concerns with eventual waste disposal and record archiving. In order to minimize liability, the Group must monitor and supervise the contractors' procedures to assure that the hazards of handling the substances being tested are controlled. Thus, baseline safety evaluations of each facility contracted by NTP are updated routinely to ensure human health is not jeopardized. In addition, chemical monitoring and industrial hygiene surveys for specific chemicals, periodic review of training needs at the contract labs, and formulations of standards, guidelines and safety plans were completed (Dr. Walters).
- A study is being conducted to compare the sensitivities of carcinogenicity test models using post-weaning lifetime exposure only to those using in utero, perinatal, and post-weaning lifetime exposure. The test chemicals are phenytoin (diphenylhydantoin), ethylenethiourea, and polybrominated biphenyls (Firemaster FF-1®) (Dr. Chhabra).
- Prechronic and chronic inhalation studies are being conducted to compare the toxic and carcinogenic potentials of three nickel compounds (nickel oxide, nickel sulfate, nickel subsulfide) with differing degrees of solubility. Although nickel is a recognized human carcinogen, the specificities of the biological effects of the various salt forms are not presently clear (Dr. Dunnick).
- Toxicity studies are being conducted on "PUVA" therapy (psoralens and UV-A light), a therapeutic regimen used to treat psoriasis and vitiligo in humans. This combination is being studied in Fischer 344 rats and hairless HRA/skh mice, surrogates for humans receiving various psoralens and light exposures (Dr. Dunnick).
- As part of a larger study, the NTP continues to test class representatives of benzidine dyes for carcinogenic effects (Dr. Mennear).

- The toxicity of intravenously administered Vitamin E and polysorbate 80 and 20 in the neonatal rabbit (cesarian delivered) is being studied. The information obtained will be of value for the safe use of this type of preparation in the prophylactic treatment of retrolental fibroplasia and respiratory distress syndrome in the premature human (Dr. Abdo).
- Under an interagency agreement with the EPA, the NTP is identifying and conducting the appropriate research and testing to eliminate "data gaps" for chemicals commonly found in waste disposal sites (Dr. Yang).
- The technical effort of encapsulating volatile and/or reactive test chemicals in non-toxic microcapsules is underway. Microencapsulation will allow such chemicals to be administered experimentally as feed admixtures instead of by gavage (Dr. Jameson).
- Studies are being conducted to evaluate the repeatability and validity of short-term in vitro tests for predicting teratogenic potential in mammals. Two complementary in vitro tests (inhibition of ascites Mouse Ovarian Tumor [MOT] cell attachment, and inhibition of growth of Human Embryonic Palatal Mesenchymal [HEPM] cells) are being evaluated for their utility as predictors of teratogenic response (Dr. Chhabra).
- Chemical compounds are maintained in repositories which are currently under study or which have completed testing in the various NTP programs including CTEB and CGTB. Over 1900 individual chemicals from these testing programs are stored and maintained in these repositories (Dr. Jameson).
- A class study of the three mononitrotoluene isomers is being initiated to determine whether predictions regarding the carcinogenic potential of each isomer that are based on extensive metabolism, in vivo, and in vitro studies with dinitrotoluene and mononitrotoluene isomers can be verified (Dr. Bristol).

Intramural Research Highlights: Intramural research is conducted at the NIEHS by CTEB staff and collaborating scientists.

- The long-term health effects of short exposure to methyl isocyanate (MIC) are being examined to provide a data base on this chemical. These studies are designed to provide information in histopathologic, pulmonary function, reproductive, immunotoxic and genetic toxicity effects resulting from inhalation exposure to MIC (Dr. Bucher).
- The effects of di(2-ethylhexyl)phthalate and other hepatic peroxisome proliferators on liver structure, function and biochemistry are being evaluated to determine whether there is any causal relationship between peroxisomes and tumor development. These studies involve the monitoring of normal and abnormal oxygen reduction (Dr. Melnick).
- The characterization of mononuclear cell leukemia (MNCL) in Fischer 344 rats and the relationship between its enhanced occurrence and chemical exposure is being studied in collaboration with Drs. Dieter (CTEB) and Maronpot (CPB) (Dr. French).
- Reproductive toxicity studies of methyl dopamine, a commonly-used antihypertensive drug, are being conducted to determine effects on the male reproductive system and the viability and health of offspring (Dr. Dunnick).

- Methods for determining the dermal absorption of chemicals using in vitro systems are being developed (Dr. Eastin).
- Mechanisms of phthalate-induced male gonadal injury in rats are being studied. These studies focus on the relationships of hormonally-mediated effects and gonadal zinc deprivation to reproductive failure (Dr. Agarwal).
- The mechanism of DBCP inhibition of sperm energy metabolism is being investigated (Dr. Melnick).
- The use of microencapsulation for toxicity testing of unstable chemicals is being evaluated in bioavailability and 14-day toxicity studies (Dr. Melnick and Dr. Jameson).

Other Activities:

- Dr. Bristol: Serves as TRTP Scientific Coordinator for development and implementation of the Toxicology Data Management System (TDMS).
- Dr. Eastin: Serves on the Ad Hoc Interagency Dermatology Working Group. This group coordinates dermatology-related research throughout the government.
- Dr. Dieter: Serves as a representative on the NIEHS Animal Use Committee. He also serves as consultant for the toluene diisocyanate criteria document being written for the World Health Organization IPCS.
- Dr. Rauckman: Continues to develop the NTP Phthalate Ester Program.
- Dr. Mennear: Participated in the preparation of World Health Organization (WHO) criteria documents on trichloroethylene and diaminotoluene.
- Dr. Yang: Coordinates and plans the implementation of "Superfund" testing activities.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21001-05 CTEB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of Chemical Nephrotoxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William M. Kluwe

Acting Chief

TRTP/CTEB

NIEHS

Others: Deepak K. Agarwal

Visiting Fellow

TRTP/CTEB

NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Carcinogenesis and Toxicology Evaluation Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

.75

PROFESSIONAL

.25

OTHER

.50

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Time- and dose-dependent effects of selected nephrotoxic agents on the ultrastructure and functional and biochemical status of target and non-target cells in the kidney are evaluated to study basic mechanisms of injury to various renal cell populations. Comparisons are made between chemical structures and the types of subcellular lesions induced, or the target cells affected, to elucidate common pathophysiological sequences of chemically-induced renal cell injury.

PROJECT DESCRIPTION

METHODS EMPLOYED: At several times post-dosing and at multiple dose levels (range of non-toxic to maximally-toxic), evaluations are made of organ morphologies by light microscopy and of subcellular organization by transmission electron microscopy. Evaluations are also made at the same times of biochemical and physiological parameters indicative of the status of cell function in general and subcellular organelle (e.g., plasma membrane, mitochondria) lability more specifically. These changes are compared to alterations in total organ (kidney) function. Both in vivo and in vitro methodologies are employed, and the relationship of duration of exposure to toxic response is studied.

MAJOR FINDINGS AND PROPOSED COURSE: Many nephrotoxic organohalide compounds that selectively injure cells of the pars recta (S₃) initially cause vesiculation of the cytoplasm in the apical portion of the cell. Later-appearing morphological effects include microbody proliferation, mitochondrial swelling, increased smooth endoplasmic reticulum and aggregation of chromatin at the periphery of the nucleus. Shortly thereafter, several tubular transport processes (e.g., ions, bulk fluids) are compromised. Functional recovery follows morphological evidence of repair.

In a time-effect correlation study, hexachlorobutadiene (HCB_D) produced severe nephrotoxicity at 24 hrs after exposure with associated biochemical alterations involving various subcellular compartmental structure(s) and function(s). The earliest biochemical was detected in microsomal cytochrome P-450 1.5 hrs after dosing.

Assessments are being made of ATP concentration, mitochondrial function, pinocytotic reabsorption, lysosomal lability, endoplasmic reticulum integrity and enzymatic activities and incorporation of precursors into RNA, DNA, protein and lipid to correlate the morphological changes with biochemical effects and to suggest molecular mechanisms of action. Also, the impact of age-related changes on nephrotoxic response to chemicals is being studied. Of particular interest is the interrelationships of hepatic and renal biotransformation in toxicant activation and the role therein of cellular glutathione.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Kidney disease is a major cause of debilitation in the United States. Although the extent of chemicals as causative agents in toxic nephropathy in humans is unknown, animal studies suggest considerable susceptibility of mammalian kidneys to halogenated hydrocarbons and organic amines. Mechanistic studies are necessary to assess experimental animals as models of human response to nephrotoxics and for the extrapolation of animal safety studies to the human situation.

PUBLICATIONS

Kluwe, W.M.: Chronic Kidney Disease and Organic Chemical Exposures: Evaluations of Causal Relationships in Humans and Experimental Animals. Fund. Appl. Toxicol., 4: 889-901, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21030-03 CTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemically-induced and Spontaneous Mononuclear Cell Leukemia in Fischer 344 Rats

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: John E. French Physiologist TRTP/CTEB NIEHS

Others: Michael P. Dieter	Physiologist	TRTP/CTEB	NIEHS
Robert R. Maronpot	Pathologist	TRTP/CPB	NIEHS
Ralph Wilson	Bio. Lab. Tech.	TRTP/CPB	NIEHS

COOPERATING UNITS (if any)

Systemic Toxicology Branch/TRTP/NIEHS
Chemical Pathology Branch/TRTP/NIEHS
Program Resources Branch/TRTP/NIEHS

LAB/BRANCH

Carcinogenesis and Toxicology Evaluation Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.5

PROFESSIONAL:

1.0

OTHER

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

NTP chronic toxicity test results indicate a high incidence of rat mononuclear cell leukemia (MNCL) in both control and treated F344 rats. It is difficult to determine if chemical induction of MNCL has occurred in a chronic test because of the high incidence of spontaneous MNCL. Responses to chemical treatment have included statistically significant increases or decreases in MNCL as well as positive or negative trends with respect to dose. A transplant model for MNCL has been characterized in terms of its clinical presentation, gross pathology, cell morphology and biochemistry, histopathology, and clinical hematology and chemistry. After subcutaneous inoculation of 2×10^7 viable leukemic cells into rats, clinical symptoms, morbidity and mortality due to MNCL occur after 100 days (95 to 105 days). Clinical symptoms are usually present after 90 days. However, with this size inoculum, splenomegaly and leukocyte count increase start to occur after 65 days. The FY 1985 objective of this research project is to develop a short-term test model for assessing the effect of chemical treatment on the expression of transplanted mononuclear cell leukemia in F344 male rats.

PROJECT DESCRIPTION

METHODS EMPLOYED: Cell separation using continuous and discontinuous gradients, monoclonal antibody and immunofluorescent surface marker assays, in vitro and in vivo cell culture and propagation, cellular and serum enzyme biochemistry, clinical chemistry and hematology procedures will be utilized during the course of this project.

MAJOR FINDINGS AND PROPOSED COURSE: Mononuclear cell leukemia as a disease was characterized by body weight loss, splenomegaly, elevated white cell and reduced red cell counts, hypoglycemia, icterus, hyperbilirubinemia, and serum enzyme elevations. Atypical mononuclear cells were predominant in spleen and blood with acentric nuclei and Cremsa stained red cytoplasmic granules. Rate-limiting enzymes of carbohydrate metabolism were elevated 200-800% in enriched mononuclear cell fractions from spleen and blood. The leukemic cell line was maintained in syngeneic recipients by S.O. transfer of 2×10^7 leukemic spleen cells. Replicate experiments showed that leukemia induced at 100 days was clinically and morphologically similar to spontaneous leukemia, but the pattern of biochemical responses in spleen mononuclear cells was inversely related to enzyme elevations in spontaneously leukemic rats, with 20-85% decreases in specific activities of carbohydrate metabolizing enzymes. There was also a 35-85% decrease in spleen and blood acetylcholinesterase activity, a marker for T-cells. A dose-response study (0.1×10^7 viable leukemic cells/recipient) was performed to determine the effect of inoculum size on morbidity, mortality and tumor expression. An optimal inoculum size of $2-4 \times 10^7$ viable cells/recipient was established.

Additional research projects will include:

1. Validation of short-term test model using selected chemicals previously tested in NTP two-year chronic tests. We will initially test the hypothesis that short-term chemical treatment of MNCL transplanted F344 male rats will increase or decrease tumor (MNCL) expression and associated characteristics in correlation with the chemical tests results of the NTP chronic toxicity test (e.g., positive or negative dose-related trend).
2. Investigation of efficacy and sensitivity of selected biochemical and tumor cell markers for early prediction of leukemia incidence.
3. Qualitative and quantitative characterization of cell surface markers and receptors and karyotype of mononuclear leukemic cells using fluorescence staining with fluorescence activated cell sorter analysis.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The investigation of F344 rat mononuclear cell leukemia affords the unique opportunity to investigate a spontaneously occurring tumor and its origin, develop clonally derived cell line(s) for in vitro study of this disease, and develop an animal model for experimental chemical induction of leukemia for toxicity and test methods development. Development of a short-term test model for assessing chemical treatment effects on transplanted F344 MNCL will provide information on determining the impact of this leukemia upon the interpretation of hematopoietic tumors incidence in NTP two-year chronic toxicity test results.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21050-02 CTEB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of Microencapsulation As A Means to Administer Chemicals in Feed.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	C. W. Jameson	Head, Collaborative Resources Group	CTEB/NIEHS
	R. L. Melnick	Head, Experimental Toxicology Unit	CTEB/NIEHS
Others:	T. J. Goehl	Chemist	CTEB/NIEHS
	A. Greenwell	Technician	CTEB/NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Carcinogenesis and Toxicology Evaluation Branch

SECTION

Collaborative Resources Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.0

PROFESSIONAL

0.25

OTHER

0.75

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Microencapsulation is a process for completely enveloping tiny masses of solid particles, or liquid droplets in a protective coating which separates the substance from its environment. The use of microencapsulated chemicals for toxicology studies presents a number of advantages, i.e., it permits testing volatile or chemically reactive compounds in the animal diet, minimizes problems with palatability, etc. Volatile and/or reactive chemicals have been encapsulated using a gum/sorbitol matrix and determined to be stable when mixed with rodent feed. Relative bioavailability of the microencapsulated trichloroethylene compared to the neat test material indicates no significant difference in absorption after oral administration. Feeding studies using the microencapsulated trichloroethylene, 1,1,1-trichloroethane and citral are planned.

PROJECT DESCRIPTION

METHODS EMPLOYED: Current analytical chemical methods utilizing conventional gas chromatography and sample extraction methods are utilized.

MAJOR FINDINGS AND PROPOSED COURSE: Trichloroethylene (TCE), 1,1,1-trichloroethane and citral are volatile liquid chemicals which the TRTP is interested in testing by an oral route other than gavage. In order to incorporate the chemicals into the feed in a stable form they were microencapsulated using a gelatin and sorbitol matrix. The resultant microcapsules were determined to be stable upon extended storage and also when mixed with rodent feed. (This work was conducted by Midwest Research Institute under contract No. N01-ES-95615.)

Relative bioavailability of microencapsulated TCE suspended in corn oil versus neat TCE dissolved in corn oil by gavage was investigated. Blood level determinations indicate no significant difference in absorption of TCE from the gut of Fischer 344 rats between the microencapsulated and neat test material.

Future studies will include bioavailability studies for 1,1,1-trichloroethane and citral and 14 day feeding studies of Fischer F344 rats and B6C3F₁ hybrid mice using the microencapsulated test material. Blood levels of trichloroethylene and/or its metabolite trichloroethanol will be determined to establish absorption of test chemical. In addition, analysis of feed samples for test chemical will be performed to determine extent of degradation of microcapsules under actual dosing conditions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The objective of this study is to explore the effect microencapsulation has on the absorption and toxic response to test chemicals. These studies will assist in evaluating the feasibility of microencapsulation as an alternative to gavage for the oral administration of volatile and/or reactive test materials.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21061-03 CTB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Interactions Between Chemical Dose and Toxicity		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> <div style="width: 33%;"> PI: William M. Kluwe Others: Robert R. Maronpot Jerry Hardisty </div> <div style="width: 33%;"> Acting Chief Pathologist Pathologist </div> <div style="width: 33%;"> TRTP/CTB NIEHS TRTP/CPB NIEHS Environmental Pathology Labs, Raleigh, NC </div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Carcinogenesis and Toxicology Evaluation Branch		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS .625	PROFESSIONAL .50	OTHER .125
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 33%;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div style="width: 33%;"> <input type="checkbox"/> (b) Human tissues </div> <div style="width: 33%;"> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided) <p> Several <u>organohalide</u> compounds are metabolized to <u>reactive intermediates</u> presumed to be the ultimately toxic molecules. The <u>reactive metabolites</u> are <u>detoxified</u> by reacting with tissue <u>non-protein sulphhydryls (NPS)</u>, and acute toxicity occurs only when NPS have been <u>depleted</u> below a critical level. Upon prolonged chemical exposure, however, a dynamic state exists between chemical metabolism, NPS depletion, NPS synthesis, and lesion development. Whether or not the same relationship exists between tissue NPS concentrations and the development of lesions in a chronic exposure situation as in an acute one is being evaluated. Also, the organ-specificity and species-specificity of these phenomena are being studied. </p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: Rodents are treated for 1, 5, 10 or 60 days with one of several organohalide compounds (e.g., bromobenzene, vinyl chloride, ethylene dibromide) by gavage or inhalation. At representative times post-exposure, the animals are killed and tissues are evaluated for morphological changes and for NPS contents and other biochemical parameters. In some instances, the time-dependent dispositions and distributions of the test chemicals are monitored.

MAJOR FINDINGS AND PROPOSED COURSE: A single exposure to bromobenzene produces a dose-dependent depletion of hepatic NPS followed by a "rebound" to higher than normal levels. Hepatotoxicity occurs only when hepatic NPS are depleted below 40% of normal. Following multiple exposures, a higher steady-state concentration of hepatic NPS is achieved. Although bromobenzene still depletes hepatic NPS, the NPS concentration apparently does not fall below a "critical" level and hepatotoxicity does not develop. The mechanism and limits of this protective change in response to prolonged bromobenzene exposure are currently being studied.

Organohalides supplied by inhalation have a lower propensity for depleting hepatic NPS, but a greater propensity for depleting non-liver NPS (e.g., lung). However, animals exposed multiply still tend to exhibit lesser toxic changes in tissue morphology than those exposed singly.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A better understanding of the dynamic nature of dose-response relative to NPS depletion in chronic studies will enhance our ability to properly choose doses for chronic studies. Similarly, exploration of the interrelationships between dose, NPS and injury in chronic exposure situations may help clarify the appropriateness (or inappropriateness) of high dose to low dose extrapolations for chronic studies involving chemicals exhibiting non-linear pharmacokinetics.

PUBLICATIONS

Kluwe, W.M., Maronpot, R.R., Greenwell, A. and Harrington, F.W.: Dynamic Interactions Between Bromobenzene Dose, Glutathione Concentrations and Organ Toxicities in Single and Repeated Treatment Studies. Fund. Appl. Toxicol., 4:1019-1028, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21062-03 CTEB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) Mechanisms of Phthalate Ester Toxicities in Mammalian Species		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI: Ronald L. Melnick	Chemist	TRTP/CTEB NIEHS
Others: Deepak K. Agarwal	Visiting Fellow	TRTP/CTEB NIEHS
James C. Lamb, IV	Biologist	TRTP/STB NIEHS
Robert R. Maronpot	Pathologist	TRTP/CPB NIEHS
Scott Eustis	Pathologist	TRTP/CPB NIEHS
Elmer J. Rauckman	Expert	TRTP/CTEB NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Carcinogenesis and Toxicology Evaluation Branch		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.365	.865	.50
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided) <p>Phthalate esters are plasticizers incorporated into nearly all plastic materials. The biochemical and ultrastructural effects of di(2-ethylhexyl)phthalate (DEHP) and related chemicals are being studied in order to assess potential mechanisms of phthalate ester toxicity.</p> <p>Since DEHP and other phthalates are also male chemosterilants and teratogenic in mice, studies are being conducted to determine the role of zinc in the pathophysiology of these reproductive effects and to discern no-observed toxic effect levels.</p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: For the reproductive studies, male rats treated with DEHP or butyl benzyl phthalate (BBP) are mated to determine fertility, and assessments are made of male gonadal structure, function and biochemistry. The role of dietary and tissue zinc in enhancing or ameliorating the gonadal effects of DEHP are monitored.

For other studies, the effects of BBP on bone marrow function and circulating blood compounds are evaluated.

MAJOR FINDINGS AND PROPOSED COURSE: Low dietary zinc was found to exacerbate DEHP gonadal toxicity in male rats. This was accompanied by a selective loss of zinc from the testis and epididymis, suggesting that DEHP produces testicular atrophy via zinc deprivation. Bilateral vasectomy failed to block the DEHP-induced elevation in urinary zinc, suggesting that DEHP may inhibit uptake of zinc into the testis, perhaps via chelation. Experiments elucidating alternative theories of perturbation of hormonal regulation leading to testicular atrophy, zinc depletion and reproductive dysfunction did not provide conclusive answers, however, hampered transport of zinc in testis due to afflicted metallothioneins may provide a mechanism of zinc depletion from testicular tissue.

Exposure of male rats of 1.5, 4, 15, and 30 months of age revealed age-related sensitivity of rats to DEHP. Gonadotoxicity was pronounced in young rats of 1.5 months of age but hypolipidemia or hepatomegaly was not influenced by age. Rate of peroxisomal proliferation by DEHP was less marked in older rats suggesting that, with the increased age, rats become less responsive to toxic challenge than young or mature rats.

Butyl benzyl phthalate causes generalized gonadal atrophy in male rats accompanied by a decreased serum concentration of testosterone (FSH and LH increased), suggesting a cause and effect relationship between hormonal changes and tissue atrophy. This compound also reduces the cellularity of the bone marrow after 14 days of treatment, indicating hematotoxic potential.

Subchronic studies are currently being conducted with butyl benzyl phthalate. The butyl benzyl phthalate study in rats will ultimately include a 2-year chronic exposure experiment. Comparative studies on other effects of DEHP, di(2-ethylhexyl)adipate and similar compounds will be conducted in parallel with chronic bioassays for evaluating their mechanisms of carcinogenic activity.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Phthalate ester plasticizers are ubiquitous contaminants of the general environment, and leachable components of most plastic consumer products. The frequent exposure of humans to these substances and the serious toxic effects (e.g., carcinogenesis, teratogenesis, sterility) associated with their prolonged use in rodents necessitates a clear understanding of their toxic potential. This task is best accomplished through an integrated program of general testing for toxicity and elucidation of mechanisms for specific toxic effects.

PUBLICATIONS

Agarwal, D.K., Maronpot, R.R., Lamb, J.C., IV, and Kluwe, W.M.: Adverse effects of butyl benzyl phthalate on the reproductive and hematopoietic systems of male rats. Toxicology (in press), 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21063-03 CTEB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Tests for the Detection and Monitoring of Chemical-Induced Pulmonary Damage

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Rajendra S. Chhabra	Supv. Pharmacologist	TRTP/CTEB	NIEHS
Others: Gary E.R. Hook	Research Chemist	LPFT	NIEHS
Paul Nettesheim	Chief	LPFT	NIEHS
Kimeri D. Collins	Bio. Lab. Tech.	TRTP/CTEB	NIEHS

COOPERATING UNITS (if any)

Biochemical Pathology Group, LPFT

LAB/BRANCH

Carcinogenesis and Toxicology Evaluation Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0.8

PROFESSIONAL

0.1

OTHER

0.7

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The objectives of this project are to develop procedures for (1) determining the potential of agents in causing lung damage and (2) detecting pulmonary injury and monitoring that injury as it progresses towards or away from the disease state. Methods for the estimation of injury potential involves the use of purified cell populations isolated from the lungs and the reaction of those cells to toxic agents. Methods for the detection of pulmonary injury involve the use of pulmonary lavage effluents and the identification of markers of cellular injury. Two cell populations have been isolated from the lungs of rabbits. Clara cells from the airway epithelium have been isolated and purified to as much as 95% with 97% viability as measured by exclusion of trypan blue. Type II cells have also been isolated and purified to as much as 85% with 88% viability. Incubation of clara cells and Type II cells with naphthalene revealed that Type II cells were considerable more sensitive to the toxicity of this agent than Clara cells. Incubation of cells with naphthalene (1mM) for a period of one hour resulted in a 20% loss in viability of the Type II cells whereas Clara cells and alveolar macrophages were unaffected. Lavage effluents from the lungs of rats treated with silica by intratracheal injection showed elevated levels of some biochemical parameters. Increases were all dose- and time-dependent. These investigations indicate that isolated cells could be used to test the cellular toxicity of chemical agents and that lavage effluents from the lungs of animals exposed to toxic agents may be used for the detection of pulmonary lung injury.

COMPLETED

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to develop procedures for: (1) determining the potential of agents in causing lung damage and (2) detecting pulmonary injury and monitoring that injury as it progresses towards or away from the disease state.

METHODS EMPLOYED: Methods for the estimation of injury potential involves the use of purified populations of cells isolated from the lungs and the reactions of those cells to toxic agents. Methods for the detection of pulmonary injury involve the use of pulmonary lavage effluents and the identification of markers of cellular injury.

MAJOR FINDINGS AND PROPOSED COURSE: We hypothesize that cells isolated from the lungs react with agents in a manner reflective of the ability of those agents to damage those cell populations in vivo and, therefore, such isolated cell populations could be used to predict the pulmonary injury capability of those agents. We further hypothesize that damage to cells of the pulmonary epithelium will result in the release of cellular and cell-specific constituents into the pulmonary extracellular lining that could be used for the detection of injury for the monitoring of the condition of specific cell populations in the lungs. Our objectives have been to isolate Clara cells, Type II cells and alveolar macrophages in highly purified forms and develop methods for their maintenance under conditions of in vitro cultivation that could provide a means for the study of the interactions between those cells and a few well characterized pulmonary toxins. Incubation of Clara cells, Type II cells and alveolar macrophages with 1 mM naphthalene did not affect the viability of Clara cells or alveolar macrophages isolated from the lungs of rabbits; however, Type II cells over a course of 1 hour showed a 20% loss in viability as measured by exclusion of Trypan Blue. These results demonstrate that the isolated cells could be used to detect pulmonary toxicants such as naphthalene. In development of methods for the detection of lung damage we have investigated the silica model as a means of inducing dose-related lung injury. We have identified several enzymes whose presence in the extracellular phase of pulmonary lavage effluents appears indicative of cellular injury. β -N-acetylglucosaminidase and soluble alkaline phosphatase are components of the pulmonary extracellular lining and both are increased more than 10-fold 28 days following a single intratracheal injection of silica (50 mg) into the lungs of rats (250 g). In response to silica, the permeability of the blood/air barrier was increased to only a small extent as evidenced by the appearance of a few high molecular weight serum proteins in lavage effluents; however, integrity of the barrier was maintained in spite of increases in the protein content of the lavage effluents. Five new proteins appeared in lavage effluents following dosing of rats with silica via the tracheal route as evidenced by two-dimensional polyacrylamide gel electrophoresis. These five proteins had molecular weights of 123,000, 123,000, 117,000, 93,000, and 80,000 daltons were detectable in lavage effluents from the lungs of silica-treated rats but not from untreated rats. These proteins are potentially useful in detecting and monitoring silica-induced lung injury.

In the next year several methods will be developed for the detection of injury in the cell populations that have been purified from the lungs. These methods will include protein biosynthesis, release of ^{51}Cr , and the measurement of mitochondrial functions as a means of expanding the concept of cellular injury. These methods will be used in conjunction with other pulmonary toxins known to induce injury to either Clara cells or Type II cells. Methods for the detection of pulmonary injury will focus on the nature and origins of proteins whose presence in lavage effluents is dependent upon epithelial damage.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Many chemical and particulate agents are injurious to the lungs and the number of those agents grows daily. There is great need for sensitive methods that can detect and monitor pulmonary damage induced by such agents. An even greater need exists for the development of methods that can predict the capacity of an agent for damaging the lungs. The approach that we have taken may lead to the development of methods for testing the potential of a chemical agent to produce lung injury and thereby avoid many costly and time consuming trial and error inhalation experiments currently being used. We do not propose that in vitro experiments with isolated cell populations could ever completely substitute for the whole animal model but isolated cell populations could provide a rapid means for screening many chemicals and identifying those compounds potentially toxic to the lungs.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21067-01 CTEB																								
PERIOD COVERED October 1, 1984 to September 30, 1985																										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Inhalation Toxicity Studies on Methyl Isocyanate in Rats and Mice																										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: John R. Bucher</td> <td style="width: 40%;">Chemist, CTEB</td> <td style="width: 30%;"></td> </tr> <tr> <td>Others: B.A. Schwetz</td> <td>Chief, STB</td> <td>TRTP/NIEHS</td> </tr> <tr> <td>E.E. McConnell</td> <td>Acting Director, TRTP</td> <td>TRTP/NIEHS</td> </tr> <tr> <td>M.D. Shelby</td> <td>Head, Mammalian Mutagenesis, CGTB</td> <td>TRTP/NIEHS</td> </tr> <tr> <td>M. Luster</td> <td>Head, Immunotoxicology, STB</td> <td>TRTP/NIEHS</td> </tr> <tr> <td>B. Gupta</td> <td>Staff Pathologist, CPB</td> <td>TRTP/NIEHS</td> </tr> <tr> <td>C. Jameson</td> <td>Head, Collaborative Resources</td> <td>TRTP/NIEHS</td> </tr> <tr> <td>C. Richter</td> <td>Chief, Comparative Medicine Branch</td> <td>NIEHS</td> </tr> </table>			PI: John R. Bucher	Chemist, CTEB		Others: B.A. Schwetz	Chief, STB	TRTP/NIEHS	E.E. McConnell	Acting Director, TRTP	TRTP/NIEHS	M.D. Shelby	Head, Mammalian Mutagenesis, CGTB	TRTP/NIEHS	M. Luster	Head, Immunotoxicology, STB	TRTP/NIEHS	B. Gupta	Staff Pathologist, CPB	TRTP/NIEHS	C. Jameson	Head, Collaborative Resources	TRTP/NIEHS	C. Richter	Chief, Comparative Medicine Branch	NIEHS
PI: John R. Bucher	Chemist, CTEB																									
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C. Jameson	Head, Collaborative Resources	TRTP/NIEHS																								
C. Richter	Chief, Comparative Medicine Branch	NIEHS																								
COOPERATING UNITS (if any) Pulmonary Physiology Testing Laboratory, EPA																										
LAB/BRANCH Carcinogenesis and Toxicology Evaluation Branch																										
SECTION Experimental Toxicology Unit																										
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																										
TOTAL MAN-YEARS: <div style="text-align: center;">4</div>	PROFESSIONAL: <div style="text-align: center;">2</div>	OTHER: <div style="text-align: center;">2</div>																								
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>																										
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Following the release of methyl isocyanate (MIC) from the Union Carbide plant in Bhopal, India, and the subsequent deaths of between 2 and 4,000 people, the Department of State, and the World Health Organization requested that the NTP examine the long term health effects of short exposures to MIC. In response, the NTP and NIEHS assembled staff with expertise in inhalation, reproductive, immuno-, genetic and general toxicology, along with staff experienced in pulmonary and general pathology, to design and implement studies which would provide a data base on this chemical. Animal studies were initiated in March of 1985 with exposure to MIC vapors accomplished in the Building 14 inhalation facility by personnel employed by Northrop, under contract N01-ES-4-5044. Rats and mice of both sexes were exposed to various concentrations of MIC for either two hours on one occasion, or for six hours on four consecutive days. Studies of complete animal histopathology were performed immediately following the exposures and at periodic intervals during the subsequent 90 days. Pulmonary effects were examined by light and electron microscopy, and were correlated with results of pulmonary function tests performed by the Pulmonary Physiology Testing Laboratory at EPA. Reproductive effects were examined by mating trials, and by evaluation of offspring from late term pregnant mice exposed on gestation days 14-17. Evaluation of immunotoxicity included tests of humoral and cell mediated immunity. Genetic toxicity evaluations included a variety of in vitro assays, cytogenetic assays in vivo, and determinations of micronuclei, and dominant lethal assays in exposed mice. </p>																										

PROJECT DESCRIPTION

METHODS EMPLOYED: The following studies were designed and implemented in response to a request to the NTP from the Department of State, and the World Health Organization for animal toxicity data on methyl isocyanate (MIC). This request followed the industrial accident in Bhopal, India in which between 2,000 and 4,000 people were killed, and many thousands more injured due to the release of 70 to 90,000 lbs of MIC from a Union Carbide agricultural chemical plant.

Rats and mice are being exposed to MIC by inhalation for short periods of time (from a single 2 hour exposure, to 4 days of 6 hours per day) in the Building 14 inhalation facility. Exposures are being carried out by Northrop personnel under contract #N01-ES-4-5044. Histopathologic changes, in particular those occurring in the respiratory system and eyes, are being assessed both immediately following the exposures, and during a subsequent 90 day period. The lung, bronchi and nasal cavity are being examined by light and electron microscopy, and pulmonary function measurements are being carried out on male rats in the EPA Pulmonary Physiology Testing Laboratory. Studies of the potential for MIC to influence the reproductive system include mating trials and evaluation of litters from late term pregnant mice exposed to MIC on gestation days 14-17. The potential for MIC to cause immunotoxicity is being assessed using tests of both humoral and cell mediated immunity. These include functional assays as well as examination of bone marrow differentials. The genetic toxicity of MIC is being evaluated in both a battery of in vitro assays, and in dominant lethal and micronucleus determinations in vivo. The potential for short term exposure to MIC to cause cancer is being addressed through exposure of both sexes of rats and mice to two hour inhalations of various concentrations of MIC, followed by a two year hold, and ultimate assessment of tumor incidence.

MAJOR FINDINGS AND PROPOSED COURSE: At this point we do not yet have results which can be reported, but we expect by early fiscal year 1986 to have complete results of genetic toxicity studies, with the immunotoxicity and reproductive toxicity results appearing somewhat later.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The significance of this program of research for the 200,000 people who were exposed to MIC is clearly evident. The long term prognosis for resolution of pulmonary injuries; decisions regarding reproductive choice; and the probable influence of MIC exposure on resistance to infectious disease are but a few of the subjects which our studies are designed to address. Beyond this, the significance of these studies to the NTP and NIEHS lies in the fact that they demonstrate the Program can respond to a disaster of this type by providing comprehensive toxicity data within a period of time short enough to be used in planning for the long term health needs of the victims.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21076-02 CTB																																
PERIOD COVERED October 1, 1984 to September 30, 1985																																		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cellular Biochemistry Studies on Chemical Selected for Evaluation by NTP																																		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: Michael P. Dieter</td> <td style="width: 20%;">Physiologist</td> <td style="width: 20%;">TRTP/CTEB</td> <td style="width: 20%;">NIEHS</td> </tr> <tr> <td colspan="4">Others: Ralph Wilson</td> </tr> <tr> <td>Gary A. Boorman</td> <td>Bio. Lab. Tech.</td> <td>TRTP/CPB</td> <td>NIEHS</td> </tr> <tr> <td>Michael I. Luster</td> <td>Pathologist</td> <td>TRTP/CPB</td> <td>NIEHS</td> </tr> <tr> <td>Linda S. Birnbaum</td> <td>Immunologist</td> <td>TRTP/STB</td> <td>NIEHS</td> </tr> <tr> <td>John E. French</td> <td>Pharmacologist</td> <td>TRTP/STB</td> <td>NIEHS</td> </tr> <tr> <td>Robert R. Maronpot</td> <td>Physiologist</td> <td>TRTP/CTEB</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>Pathologist</td> <td>TRTP/CPB</td> <td>NIEHS</td> </tr> </table>			PI: Michael P. Dieter	Physiologist	TRTP/CTEB	NIEHS	Others: Ralph Wilson				Gary A. Boorman	Bio. Lab. Tech.	TRTP/CPB	NIEHS	Michael I. Luster	Pathologist	TRTP/CPB	NIEHS	Linda S. Birnbaum	Immunologist	TRTP/STB	NIEHS	John E. French	Pharmacologist	TRTP/STB	NIEHS	Robert R. Maronpot	Physiologist	TRTP/CTEB	NIEHS		Pathologist	TRTP/CPB	NIEHS
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Robert R. Maronpot	Physiologist	TRTP/CTEB	NIEHS																															
	Pathologist	TRTP/CPB	NIEHS																															
COOPERATING UNITS (if any) Systemic Toxicology Branch, TRTP Chemical Pathology Branch, TRTP Program Resources Branch, TRTP																																		
LAB/BRANCH Carcinogenesis and Toxicology Evaluation Branch																																		
SECTION																																		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																																		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5																																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The effect of inorganic or organic metals and metal complexes is of particular interest to the NTP because of their prevalence in drinking water and industrial processes, use as constituents in anticancer drugs, and their diverse target organ toxicities.</p> <p>Mechanisms of cellular immunotoxicity were studies in rats or mice exposed to mercuric chloride, nickel sulfate or titanocene dichloride. Tissue accumulation of these metals in target organs indicated the sensitivity of the biochemical assays to evaluate target organ toxicity often preceeded gross, microscopic or clinical methods. Development of animal models to study Fischer rat leukemia revealed tumor markers for this disease that can be used to distinguish between age-induced and chemically-enhanced leukemogenesis. Urinary enzyme responses to nephrotoxic chemical insult were evaluated to use as a model to predict renal toxicity in chronic studies. Enzymatic method development to enhance stability, sensitivity, and efficiency of 7-ethoxycoumarin-ortho-demethylase was initiated to evaluate MFO-initiating activity of chemicals in minute amounts of rodent tissue.</p>																																		

PROJECT DESCRIPTION

METHODS EMPLOYED: Current biochemical and analytical chemistry methods utilizing UV spectrophotometry, centrifugal analysis separation, radio-enzymatic analysis, and high-pressure liquid chromatography are utilized.

MAJOR FINDINGS AND PROPOSED COURSE:

Mercury Studies -- Urinary enzyme responses to long-term chemical exposure by gavage were evaluated in rats and mice after 15 months treatment. The 4 enzymes selected out of the 7 evaluated earlier were assayed to determine their utility for detecting chronic renal toxicity. These enzymes included lactate dehydrogenase, aspartate aminotransaminase, alkaline phosphatase, and gamma-glutamyl transpeptidase. Histological evidence of pathological responses will be correlated with biochemical responses.

Titanocene Dichloride (Leukemia Research Program) -- Titanocene accumulation in tissues was evaluated in gavaged rats, including 15 and 18 month intervals, and at the 24 month chronic study termination. Spleen samples were collected from contract laboratory animals and mononuclear cell samples separated and frozen for later enzyme analyses. Blood samples were evaluated for evidence of leukemia. Glucose metabolizing enzymes and acetylcholinesterase will be evaluated in the spleen mononuclear cells to correlate with histopathological evidence and to evaluate their utility as marker enzymes for leukemia. Publications of this work are appended and indicate that glucose-6-phosphate dehydrogenase and maleate dehydrogenase are early indicators of leukemogenesis in leukemic mononuclear cells, and that acetylcholinesterase is the earliest biochemical prognosticator of leukemia among the enzymes evaluated; decrease in this enzyme activity indicated loss of T-cells from the mononuclear cell population. This response occurred prior to hematological, biochemical, or histological evidence of leukemia in the F344 rat.

Continuation of the leukemia research project in F344 rats will continue, and will include an evaluation of the potency of frozen spleen mononuclear cells prepared from serial donors with leukemia, and an evaluation of the type of acetylcholinesterase variant that is respondent in the F344 rat. An evaluation of chemicals that are known to have caused an increased incidence of leukemia in chronic tests will be made in the rat leukemia model to determine the efficacy of this system for detecting chemical leukemogenesis in a 60-90 day period. The inverse relationship between hepatocellular tumors and leukemias in the F344 rat will be investigated in the rat leukemia model to determine potential release of inhibitory factors from the hepatocellular tumors.

Nickel Sulfate -- B6C3F₁ female mice were given doses of 0, 1, 5, and 10 g/L nickel sulfate in their drinking water for up to 24 weeks to determine the plateau of nickel accumulation in whole blood samples taken at 4, 8, 15, and 24 weeks. Water consumption, body weight gain, organ weight changes, and mortality was recorded at selected intervals. Tissue samples were collected in mice given nickel-dosed drinking water for 15 weeks to determine metal accumulation in liver, kidney, and immunoresponsive organs (bone marrow, spleen and thymus)

since there is evidence of nickel immunotoxicity. The biochemistry and immunoresponses of the same organs will be evaluated after nickel sulfate exposure to determine the nature and extent of the immunotoxic effects.

Enzyme Method Development -- The assay of 7-ethoxycoumarin-ortho-demethylase (7-ECODE) by current methodology involves a two-phase extraction and centrifugation step following incubation of substrate and product to separate the two, and subsequent evaluation in a spectrofluorometer within minutes to prevent deterioration of the short-lived product. Methods are being developed to assay the product in an alkaline-salt mixture that is completely stable, to bypass the extraction-centrifugation steps by utilizing the HPLC, and to improve the sensitivity of the assay > 100-fold by utilizing the HPLC detector in a sealed system. Efficiency is further enhanced by utilizing an automatic injector for sample delivery. The method is being developed to assay 7-ECODE enzyme activity in bone marrow, spleen and liver samples of mice treated with MFO-activating chemicals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Information regarding the cellular toxicity of each chemical may be useful in therapeutic intervention and will provide another sensitive measure of potential chemical hazards to man. Discrimination between age-induced leukemia and chemically-enhanced leukemia will increase the sensitivity and accuracy of tests for chemical leukemogens in the F344 rat.

PUBLICATIONS

Dieter, M.P., Maronpot, R.R., and French, J.E.: Comparison of the morphology and carbohydrate metabolism of mononuclear cells from Fischer 344 rats with either spontaneous or transplanted leukemia. Cancer Research, (in press, 1985).

Dieter, M.P., Maronpot, R.R., and French, J.E.: Biochemical markers for Fischer rat leukemia in a cell transplant model. Cancer Detection and Prevention (in press), 1985.

Dieter, M.P., Wilson, R., and Birnbaum, L.S.: Age-related changes in glucose metabolizing enzymes in spleen, thymus, and pulmonary lavage cells from F344 rats. Mech. Aging Dev., 26: 287-297, 1984.

Luster, M.I., Boorman, G.A., Korach, K.S., Dieter, M.P., and Hong, L.: Mechanisms of estrogen-induced myelotoxicity: evidence of thymic regulation. Int. J. Immunopharm. 6: 287-297, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21078-02 CTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Bioavailability and Toxicity Studies of Microencapsulated Chemicals

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Ronald L. Melnick Chemist TRTP/CTEB NIEHS

Others: C.W. Jameson Chemist TRTP/PRB NIEHS

T. Goehl Chemist TRTP/PRB NIEHS

J.H. Mennear Pharmacologist TRTP/CTEB NIEHS

COOPERATING UNITS (if any)

Midwest Research Institute, Kansas City, MO
Program Resources Branch, TRTP

LAB/BRANCH

Carcinogenesis and Toxicology Evaluation Branch

SECTION

Experimental Toxicology Unit

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2

PROFESSIONAL

1

OTHER

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Trichloroethylene (TCE) and 2,6-xylidine, two volatile chemicals, have been separately encapsulated in gelatin-sorbitol microcapsules. These formulations have been shown to provide sufficient stability to the chemicals so that they may be useful in dosed-feed toxicology studies. The objectives of this project are to compare the rates and extents of absorption of neat and microencapsulated chemicals in rats and mice, and to evaluate the feasibility of using microencapsulation as a means of incorporating unstable test chemicals into rodent feed for toxicology studies. The rates and extents of absorption of TCE, prepared either as a suspension of microencapsulated TCE in corn oil or as a solution of neat TCE in corn oil, administered by gavage to male Fischer 344 rats have been studied. Similar studies in B6C3F₁ mice are being developed. The toxicity of microencapsulated TCE in rats and mice is being studied.

PROJECT DESCRIPTION

METHODS EMPLOYED: Bioavailability studies of neat and microencapsulated TCE administered by gavage in corn oil have been performed on venous-cannulated male F344 rats. Blood samples were drawn at specific time points up to 24 hours and analyzed for TCE content by gas chromatography. The 14-day toxicity studies include: 1) special in vivo and in vitro evaluations for TCE induced renal toxicity, 2) clinical chemistry measurements, and 3) liver and kidney histopathologic evaluations. Determinations of TCE concentration in the feed during and at the end of the study are being made to verify the stability of the dosed-feed mixtures.

MAJOR FINDINGS AND PROPOSED COURSE: Stabilized preparations of microencapsulated TCE and microencapsulated 2,6-xylydine have been prepared. Loss of either chemical stored in uncovered petri dishes at 24°C and 50% relative humidity for 2 weeks was less than 2%. Homogeneous feed blends of microencapsulated TCE and microencapsulated 2,6-xylydine have also been prepared. The bioavailability of microencapsulated TCE was found to be comparable to the bioavailability of neat TCE in rats. A 14-day dosed-feed toxicity study of microencapsulated TCE and unencapsulated (neat) TCE in rats has been performed. Dose-related increases in organ (liver and kidney) weight/body weight ratios, in individual cell necrosis in the liver, and in hepatic microsomal and peroxisomal enzyme activities were found in rats treated with neat or microencapsulated TCE. A similar toxicity study in mice is planned. Additional chemicals of interest to NTP are being evaluated for their stability in gelatin-type microcapsules.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Microencapsulation is a process in which a thin and continuous polymeric coating is applied onto small particulate solids or liquid droplets. For toxicology studies this process may provide a means for administering reactive, volatile, or unpalatable chemicals into animal feed. Generally, such chemicals are administered by gavage when the oral route is the intended route of exposure. Microencapsulation can provide an alternative to the gavage procedure, and would therefore eliminate potential effects associated with bolus doses, and reduce labor intensity and costs of toxicology studies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 21079-02 CTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Mechanism of Di(2-ethylhexyl)phthalate Hepatotoxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Ronald L. Melnick Chemist TRTP/CTEB NIEHS

Others: William M. Kluwe Pharmacologist TRTP/CTEB NIEHS
Deepak K. Agarwal Visiting Fellow TRTP/CTEB NIEHS
K. Tomaszewski Visiting Fellow TRTP/CTEB NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Carcinogenesis and Toxicology Evaluation Branch

SECTION

Experimental Toxicology Unit

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

2

PROFESSIONAL

1

OTHER

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

In a rodent bioassay conducted by the National Toxicology Program, di(2-ethylhexyl)phthalate (DEHP) was found to be carcinogenic for the liver in B6C3F₁ mice and F344 rats. Since DEHP also causes peroxisome proliferation, it has been suggested that the carcinogenicity of this chemical may be related to excessive peroxisomal production of H₂O₂. It is the objective of this project to examine the changes in H₂O₂ concentrations resulting from peroxisomal fatty acyl-CoA oxidation and catalase activity in livers of rats and mice treated with DEHP. Further assessment of an involvement of reactive intermediates of oxygen reduction in DEHP induced hepatotoxicity will be made from measurements of (a) activities of enzymes that eliminate toxic oxygen products (catalase, superoxide dismutase, glutathione peroxidase), (b) lipid peroxidation, and (c) superoxide anion radical production.

PROJECT DESCRIPTION

METHODS EMPLOYED: Assay procedures for measuring peroxisomal fatty acyl-CoA oxidase activity, spectrophotometrically via NAD reduction or fluorimetrically coupled to peroxidase catalyzed oxidation of scopoletin, have been developed.

MAJOR FINDINGS AND PROPOSED COURSE: The rates of liver peroxisomal palmitoyl CoA oxidase and catalase activities are increased in male F344 rats and B6C3F₁ mice treated with DEHP (2 g/kg) for 14 days. The outcome of these changes is an increase in the in vitro steady state concentrations of H₂O₂. In vivo concentrations of palmitoyl-CoA will be determined by HPLC to relate the in vitro findings to the H₂O₂ concentrations in liver cells of DEHP-treated rats and mice. Peroxisomal proliferation is also apparent in the kidneys of rats treated with DEHP.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Some groups have suggested that doses of DEHP which do not cause peroxisome proliferation would not pose a carcinogenic risk. Since the role of peroxisomes in DEHP induced carcinogenicity is speculative, it is critical that this issue be resolved. The NTP bioassay on the carcinogenicity of DEHP has stimulated many groups to initiate research activities to study the mechanism of phthalate carcinogenesis. This research project is part of NTP's effort to further characterize the toxicologic properties of DEHP.

PUBLICATIONS

Melnick, R. L. and Schiller, C. M. (1985). Effect of phthalate esters on energy coupling and succinate oxidation in rat liver mitochondria. Toxicology 34: 13-27.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 30100-06 CTEB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Toxic Effects of 1,2-Dibromo-3-chloropropane on the Urogenital System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Ronald L. Melnick Chemist TRTP/CTEB NIEHS

Others: Deepak K. Agarwal Visiting Fellow TRTP/CTEB NIEHS

Arnold Greenwell Biologist TRTP/CTEB NIEHS

Frank Harrington Bio. Lab. Tech. TRTP/CTEB NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Carcinogenesis and Toxicology Evaluation Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

.75

PROFESSIONAL

.25

OTHER

.50

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The acute and subchronic toxic effects of the pesticide 1,2-dibromo-3-chloropropane (DBCP) and structurally-related compounds are studied from functional and mechanistic viewpoints. A reported chemo-sterilant in humans, DBCP is no longer manufactured in the U.S., but its presence in ground water and on edible imports and its illegal bulk transport into certain areas of the U.S. require its further toxicological characterization. Effects of DBCP on hepatic, renal, and reproductive functions and development are evaluated at several dose levels, after various treatment regimens and under differing conditions such as age, chemical or physical stress and the like.

The mechanism of DBCP inhibition of sperm energy metabolism is being investigated.

PROJECT DESCRIPTION

METHODS EMPLOYED: Toxic effects are being studied in developing or mature male Fischer 344 rats using a variety of functional, biochemical and pathological techniques. Effects on isolated epididymal sperm are also being evaluated.

MAJOR FINDINGS AND PROPOSED COURSE: Acute intoxication with DBCP causes dose-dependent injury to the kidney, testis, epididymis and liver. Effects on the liver, epididymis and kidney appear to be reversible, but testicular damage is progressive and may be irreversible following significant acute injury. The acute effects of the DBCP metabolites epi- and alpha-chlorohydrin and -chlorolactic acid, but not with oxalic acid, another DBCP metabolite. These results suggest that DBCP, epichlorohydrin and alpha-chlorohydrin may exert their effects via a common pathophysiological mechanism. DBCP is detoxified by conjugation with hepatic glutathione, and the threshold acute toxic dose of DBCP coincides with the dose that significantly depletes hepatic glutathione. Immature rats (24 days old) are relatively resistant to the acute toxic effects, but neonates are extremely sensitive to the gonadotoxic effects of DBCP. Repeated exposure to acutely less-than-toxic DBCP doses produces a transient period of infertility in male rats, but no change in epididymal sperm number, motility or morphology. The decreased fertility appears to occur secondary to a decrease in sperm energy metabolism; the biochemical mechanism of this effect is being investigated. The basis for the extreme sensitivity of very young animals to DBCP gonadal toxicity will be studied. In vitro methods will be employed to study the biochemical lesions produced in sperm by DBCP.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Characterization of the toxic effects of DBCP and elucidation of the mechanisms of action of this and similar toxic halocarbon compounds will allow better estimates of human hazard to be made. Observance of reduced fertility at doses below those which reduce sperm number suggest that sperm counts (currently used as an index of human DBCP toxicity) may be inadequate to ensure safe human exposures. Similarities between the toxic actions of DBCP, epi- and alpha-chlorohydrin indicate the possibilities of "DBCP-like" effects for chemicals that have similar structures.

LOVELACE INHALATION TOXICOLOGY RESEARCH INSTITUTE
Albuquerque, New Mexico 87185
(IAG-222-Y01-ES-20088)

TITLE: Inhalation Study of Talc

CONTRACTOR'S PROJECT DIRECTOR: Charles H. Hobbs, D.V.M.

PROJECT OFFICER (NIEHS): Michael P. Dieter, Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1982

CURRENT ANNUAL LEVEL: \$371,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Chronic study in progress; six-month interim sacrifice completed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans.

PUBLICATIONS

Hanson, R.L., Benson, J.M., Henderson, T.R., Carpenter, R.L., Pickrell, J.A., and Brown, S.C.: Method for determining the lung burden of talc in rats and mice after inhalation exposure to talc aerosols. J. Appl. Toxicol. (in press), 1985.

LOVELACE INHALATION TOXICOLOGY RESEARCH INSTITUTE
Albuquerque, New Mexico 87185
(IAG-222-Y01-ES-30108-00)

TITLE: Prechronic and Chronic Studies of Nickel Oxide, Nickel Sulfate, and Nickel Subsulfide

CONTRACTOR'S PROJECT DIRECTOR: Charles H. Hobbs, Ph.D

PROJECT OFFICER (NIEHS): Michael P. Pieter, Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$664,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Repeated-dose study of nickel subsulfide complete; those for nickel sulfate and nickel oxide to begin soon. Work on all three compounds will proceed thorough subchronic and chronic phases.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

INTERAGENCY AGREEMENT
THE AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY
AND
THE NATIONAL TOXICOLOGY PROGRAM
IAG-222-Y03-ES-30110

TITLE: Superfund Project

PROJECT OFFICER (NIEHS): Raymond S.H. Yang, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: July 26, 1983

CURRENT ANNUAL FUNDING LEVEL: \$4,881,000

PROJECT DESCRIPTION

OBJECTIVES: Under this agreement, the NTP is participating in Public Health Service (PHS) activities related to the Comprehensive Environmental Response, Compensation and Liability Act (Superfund) by conducting toxicity testing on chemicals that were identified in priority hazardous waste sites, or otherwise released into the environment, for which adequate toxicological data are not available.

METHODS EMPLOYED: As chemicals are identified by the Environmental Protection Agency, they are referred by CDC or EPA to the Hazardous Waste Information Evaluation Subcommittee (HWIES) of the Committee to Coordinate Environmental and Related Programs (CCERP). If this Subcommittee determines that there is insufficient toxicological information on a chemical, the Subcommittee nominates it for NTP testing. The NTP will determine the appropriate tests to be undertaken on the chemicals nominated unless it has information which indicates that adequate testing is already under way or has been completed elsewhere. The results of testing will be made available in accordance with NTP established procedures.

The first group of 24 chemicals (including isomers) identified for the Superfund Project is as follows:

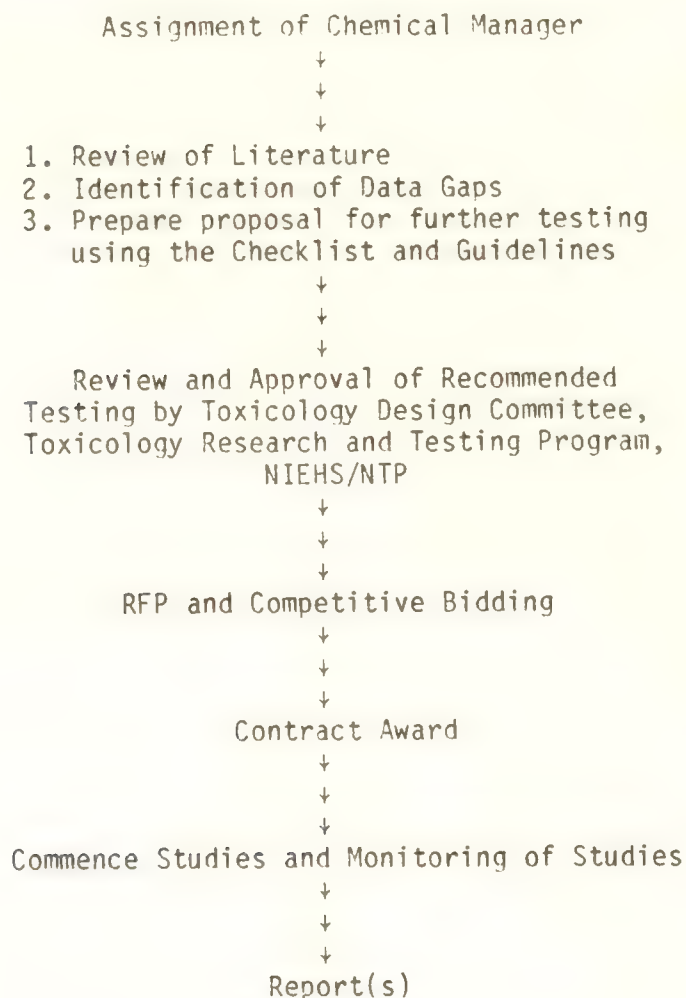
Aniline	Hexachloro-1,3-butadiene
Benzene	n-Hexane
Bromoform	Methyl Ethyl Ketone
Carbon Tetrachloride	Pentachlorobenzene
Chlorobenzenes	Phthalates
1,2-Dichloroethane	1,1,2,2-Tetrachloroethane
Dichloroethylenes	Tetrachloroethylene
Dichloromethane	Tetrahydrofuran
1,2-Dichloropropane	Toluene
Ethyl Benzene	1,1,1-Trichloroethane
Ethylene Glycol	Trichloroethylene
Hexachlorobenzene	Xylenes

MAJOR ACTIVITIES AND PROPOSED COURSE: The Superfund Project activities may be summarized in two parts:

Part I. Individual Chemical Testing:

The original list of 24 chemicals was expanded to 31 single chemicals to include some of the isomers and commercial mixtures of dichlorobenzenes and dichloroethylenes and three most widely used phthalates (diethyl-, dibutyl-, and di[2-ethylhexyl]phthalate). Each of the 31 chemicals is handled according to the Flow Chart shown below:

Individual Chemical Testing: Flow Chart



The testing status of these 31 chemicals is presented below:

STATUS A (To be reviewed by the Toxicology Design Committee [TDC]
in calendar year 1985)

Aniline
Carbon Tetrachloride
1,2-Dichlorobenzene
1,4-Dichlorobenzene
Ethyl Benzene (Cytogenetics and Salmonella are complete)
Ethylene Glycol
Hexachlorobenzene
Methyl Ethyl Ketone
Di(2-ethylhexyl)phthalate
Tetrachloroethylene
Xylene (commercial mixture of isomers)

STATUS B (Reviewed; studies deferred)

1,2-Dichloropropane (awaiting decision by EPA's Office of Toxic
Substances on proposed test ruling)

STATUS C (Studies to start in 1985)

Bromoform

In vivo cytogenetics (comparative with chloroform)

Nominated * for reproduction toxicity testing

Nominated * for immunotoxicology testing

1,2-Dichloroethane

Repeated dose (14-day) studies in rats and mice

Subchronic (13-week) studies in rats and mice

Chronic (2-year) studies after subchronic completion

1,2-cis-Dichloroethylene

Repeated dose (14-day) studies in rats and mice by microencapsulation

Subchronic (13-week) studies in rats and mice by microencapsulation

Mouse lymphoma assay

1,2-trans-Dichloroethylene

Repeated dose (14-day) studies in rats and mice by microencapsulation

Subchronic (13-week) studies in rats and mice by microencapsulation

Mouse lymphoma assay

1,2-cis- plus 1,2-trans-Dichloroethylene (1:1 Mixture)

Repeated dose (14-day) studies in rats and mice by microencapsulation

Subchronic (13-week) studies in rats and mice by microencapsulation

Dichloromethane (methylene chloride)

Behavioral teratology study

STATUS C (Studies to start in 1985 - continued)

n-Hexane

In vivo cytogenetics

2 generation reproductive toxicity testing with behavioral teratology

Repeated dose (14-day) toxicity testing in rats and mice

Subchronic (13-week) toxicity testing in rats and mice

Chronic (2-year) toxicity testing in rats and mice

Pentachlorobenzene

Nominated * for chemical disposition studies

Repeated dose (14-day) toxicity testing in rats and mice

Subchronic (13-week) toxicity testing in rats and mice

Chronic (2-year) toxicity testing (to be reviewed after completion of subchronic tests)

Nominated * for reproduction toxicity tests in mice

1,1,2,2-Tetrachloroethane

Repeated dose (14-day) toxicity testing in rats and mice

Subchronic (13-week) toxicity testing in rats and mice

Reproductive toxicity testing in rats

Nominated * for teratology testing in rats

Tetrahydrofuran

Subchronic (13-week) toxicity testing in rats and mice

Chronic (2-year) toxicity testing in rats and mice

STATUS D (Studies in Progress)

Hexachloro-1,3-butadiene

Chemical disposition

Perinatal toxicity (planned)

Chronic (2-year) toxicity (planned)

Dibutylphthalate

Reproductive toxicity (testing completed)

Diethylphthalate

28-Day dermal repeated dose in rats and mice (completed)

Chronic (2-year) dermal studies in rats and mice ** (to start - 1985)

Initiation-Promotion ** (to start - 1985)

Reproductive toxicity (testing completed)

Diethylhexyl phthalate

Reproductive toxicity (testing completed)

Butyl benzyl phthalate

26-Week subchronic dosed feed study in male rats

Dipentyl phthalate

Reproductive toxicity

STATUS D (Studies in Progress - continued)

Dipropyl phthalate
Reproductive toxicity

Toluene
Chronic (2-year) toxicity studies in rats and mice

Trichloroethylene
Reproductive toxicity testing

STATUS E (Completed Chemicals)

Benzene
No new studies recommended

1,1-Dichloroethylene
No new studies recommended
(Completed tests)

Monochlorobenzene
No new studies recommended

STATUS F (Completed Tests)

Ethyl Benzene
Cytogenetics and Salmonella are complete. To be evaluated
(Status A) for comprehensive evaluation.

* Nominated means further review will be conducted prior to final decision to conduct testing.

** Funded by NTP; not charged to Superfund.

Part II. Toxicological Studies on Chemical Mixtures:

At present, the effort on studying the chemical mixtures follows a three-prong approach:

1. Chemical Mixtures of Environmental Concern - A research proposal has been approved by the TRTP Management Committee. This proposal will be reviewed by an ad hoc committee of experts in the field of toxicological interaction studies before an RFP is issued. This proposal, which is aimed at the investigation of the combined toxic effects of long-term ingestion of some of the more frequently occurring drinking water contaminants, includes: 3-month subchronic toxicity studies on each of three mixtures of 10-12 chemicals per mixture; and investigation of possible synergistic/potentiative and/or antagonistic effects of drinking water contaminants on the oncogenicity of two known carcinogens.

2. In Vitro Toxicity Testing of Chemical Mixtures - This project is for the purpose of screening possible joint toxic actions of chemical mixtures using primary hepatocyte cultures and for investigating mechanistic aspects of cellular joint action. The studies will be conducted at the Experimental Toxicology Unit of CTEB. This project is at the stage of developing techniques for the primary hepatocyte culture.
3. Toxicological Interactions of Binary Mixtures - A proposal has been approved for the Experimental Toxicology Unit, CTEB, to conduct some pilot studies to explore the joint toxicity of binary mixtures in vivo based on known toxic mechanisms using target organs such as liver, kidney, the nervous system, and the reproductive organs.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The problem of hazardous waste in relation to environmental contamination and potential human exposure are of grave concern nationally and internationally. The activities under this Interagency Agreement represent part of a concerted effort of different government agencies to deal with a very complex problem. The scientific information generated from the NTP testing under the Superfund Project will be a contribution few other Institutions have the personnel and resources to make. Such information will be advantageous when evaluating the hazards of waste sites.

LOVELACE INHALATION TOXICOLOGY RESEARCH INSTITUTE
Albuquerque, New Mexico 87185
(IAG-222-Y01-ES-40126)

TITLE: Prechronic Studie of Azodicarbonamide

CONTRACTOR'S PROJECT DIRECTOR: Charles H. Hobbs, D.V.M.

PROJECT OFFICER (NIEHS): Michael P. Dieter, Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1984

CURRENT ANNUAL LEVEL: \$ 00.00 (Funded in FY 84 for FY 85)

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Developmental work in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

TEMPLE UNIVERSITY SCHOOL OF MEDICINE
Philadelphia, Pennsylvania 19140
(NIH-NO1-CP-15752)

TITLE: Prechronic Studies for the Bioassay of 8-Methoxypsoralen and Related Derivatives

CONTRACTOR'S PROJECT DIRECTOR: P. Donald Forbes, Ph.D.

PROJECT OFFICER (NIEHS): June K. Dunnick, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: March 31, 1981

CURRENT ANNUAL LEVEL: \$50,000

PROJECT DESCRIPTION

OBJECTIVES: This contract is designed to investigate the toxicity and/or carcinogenicity of the psoralens with and without ultraviolet light (320-400 nm [UVA]). This project will test a variety of psoralen compounds and compare their relative toxicities. The toxicity of 8-methoxypsoralen (8-MOP), 3-carbethoxypsoralen (3-CEP), 5-methylisopsoralen (5-MIP) and 5-methoxypsoralen (5-MOP) will be studied in the HRA/skh mouse. This contractor provides HRA/skh mice to other NTP contractors involved in the NTP psoralen project.

METHODS EMPLOYED: A combination exposure of psoralen and UVA treatment is given to the HRA/skh hairless mouse. Toxicologic effects are measured by skin appearance, weight gain, clinical signs, and gross and microscopic pathologic analysis of tissues.

MAJOR FINDINGS AND PROPOSED COURSE: This contractor has completed a 13-week study in the HRA/skh mouse comparing the toxicologic properties of 8-MOP, 3-CEP, 5-MIP, and 5-MOP with and without UVA light. The psoralen was delivered by a "pulsed feed" technique which minimized topical contact with the drug, and allowed for a controlled UVA light exposure 30 minutes after drug intake. 8-MOP and 5-MOP showed severe photobiologic skin toxicity when used in combination with UVA causing hyperplasia, inflammation, ulceration and dysplasia (8-MOP) or atypical nuclei (5-MOP). Little or no photobiologic skin toxicity was seen with 3-CEP and 5-MIP. There was no skin response to drug in the absence of UVA. A one year study with 8-MOP and UVA is now underway to determine tumor response.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

8-Methoxypsoralen plus UVA light (PUVA) was approved in May 1982 by the Food and Drug Administration for the treatment of psoriasis. It is estimated that approximately 3% of the United States population suffers from this disease. The NIADDK Collaboration Psoralen Study Group has found that there are three possible complications from PUVA treatment: increase in skin cancer, eye lesions, and immunologic damage. The object of the NTP animal studies is to help identify the toxic properties of PUVA therapy. This project is part of the overall mission of the National Toxicology Program to determine the toxicity of drug therapy, and to work with other branches of the government in defining and identifying toxic substances.

PUBLICATIONS

Jameson, C.W., Dunnick, J.K., Brown, R.O. and Murrill, E.: Chemical characterization of psoralens used in the NTP research projects. J. Natl. Cancer Inst., Monograph 66, 1984.

HAZLETON LABORATORIES AMERICA, INC.
Chemical and Biomedical Sciences Division
Madison, Wisconsin 53707
(NIH-N01-ES-05696-01)

TITLE: Bioassay of Two Chemicals: Methylphenidate Hydrochloride and
Riddelliine

CONTRACTOR'S PROJECT DIRECTOR: Karen M. MacKenzie, Ph.D.

PROJECT OFFICER (NIEHS): Michael P. Dieter, Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: \$2,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Methylphenidate Hydrochloride - Prechronic histopathology in progress.

Riddelliine - Prechronic tests terminated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

HAZLETON LABORATORIES AMERICA, INC.
Chemical and Biomedical Sciences Division
Madison, Wisconsin 53707
(NIH-N01-ES-05696-02)

TITLE: Bioassay of p-Nitroaniline and o-Nitroanisole

CONTRACTOR'S PROJECT DIRECTOR: Karen M. MacKenzie, Ph.D.

PROJECT OFFICER (NIEHS): Michael P. Dieter, Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: \$ 00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

p-Nitroaniline - Prechronic studies complete.

o-Nitroanisole - Prechronic studies complete.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION
Mattawan, Michigan 49071
(NIH-N01-ES-05700-01)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals for Toluene, Isoproterenol, Hydrochloride and Dimethyloldihydroxyethylene Urea

CONTRACTOR'S PROJECT DIRECTOR: Barrie Phillips, Ph.D.

PROJECT OFFICER (NIEHS): Joseph Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: August 31, 1980

CURRENT ANNUAL LEVEL: \$789,816

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: A Ninety-day subchronic study was reported for dimethyloldihydroxyethylene urea (gavage). A chronic study with toluene (inhalation) was completed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION
Mattawan, Michigan 49071
(NIH-N01-ES-05700-03)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals for
Azodicarbonamide and Isobutyl nitrite.

CONTRACTOR'S PROJECT DIRECTOR: Barrie Phillips, Ph.D.

PROJECT OFFICER (NIEHS): Joseph Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: August 31, 1980

CURRENT ANNUAL LEVEL: \$ 00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: A ninety-day subchronic study was reported for isobutyl nitrite.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION
Mattawan, Michigan 49071
(NIH-N01-ES-05700-04)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals for Carvone, Resorcinol and Diethylphthalate.

CONTRACTOR'S PROJECT DIRECTOR: Barrie Phillips, Ph.D.

PROJECT OFFICER (NIEHS): Joseph Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: October 6, 1980

CURRENT ANNUAL LEVEL: \$529,447

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Chronic gavage studies with carvone and resorcinol were completed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION
Mattawan, Michigan 49071
(NIH-N01-ES-05700-05)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals for Mercuric Chloride, Palladium Chloride and Monochloroacetic Acid.

CONTRACTOR'S PROJECT DIRECTOR: Barrie Phillips, Ph.D.

PROJECT OFFICER (NIEHS): Joseph Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: August 31, 1980

CURRENT ANNUAL LEVEL: \$2,944

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: A chronic gavage study with monochloroacetic acid was completed and reported. A chronic study with mercuric chloride was completed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION
Mattawan, Michigan 49071
(NIH-N01-ES-05700-06)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals for
Chloramphenicol, 4,4'-Diamino-2,2'-stilbenedisulfonic Acid and Cadinene.

CONTRACTOR'S PROJECT DIRECTOR: Barrie Phillips, Ph.D.

PROJECT OFFICER (NIEHS): Joseph Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch TRTP

DATE CONTRACT INITIATED: August 31, 1980

CURRENT ANNUAL LEVEL: \$422,495

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6CF₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: A chronic feeding study with 4,4'-diamino-2,2'-stilbenedisulfonic acid was completed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
BATTELLE PACIFIC NORTHWEST LABORATORIES
Richland, Washington 99352
(NIH-N01-ES-28003-01)

TITLE: Carcinogenicity Studies of Hexachlorocyclopentadiene

CONTRACTOR'S PROJECT DIRECTOR: Harvey A. Ragan, D.V.M.

PROJECT OFFICER (NIEHS): Joseph H. Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1983

CURRENT ANNUAL LEVEL: \$110,834

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: A subchronic study with hexachlorocyclopentadiene was completed and reported.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
BATTELLE PACIFIC NORTHWEST LABORATORIES
Richland, Washington 99352
(NIH-N01-ES-28003-02)

TITLE: Toxicity Studies of Bromobenzene

CONTRACTOR'S PROJECT DIRECTOR: Harvey A. Ragan, D.V.M.

PROJECT OFFICER (NIEHS): Joseph H. Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$350,238

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Subchronic gavage studies as well as 4-day inhalation studies with bromobenzene were completed and reported. A 90-day inhalation study is in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BIOASSAY SYSTEMS CORPORATION
Woburn, Massachusetts 01801
(NIH-N01-ES-28004-02)

TITLE: Prechronic Study of 4,4'-Thio-Bis(6-t-butyl-m-cresol)

CONTRACTOR'S PROJECT DIRECTOR: Indu Muni, Ph.D.

PROJECT OFFICER (NIEHS): June K. Dunnick, Ph.D., Toxicologist,
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: August, 1983

CURRENT ANNUAL LEVEL: \$54,245 and funded in previous year

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. There is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory is in the process of conducting prechronic toxicity studies with 4,4-Thio-bis-(6-t-butyl-m-cresol). These studies are performed in accordance with the NTP Master Agreement and involve dosing the animals by the feed route of exposure; noting clinical signs, body weight and food consumption during the study; and performing a gross and microscopic evaluation of animal tissues at the conclusion of the study. This laboratory is also performing clinical chemistry evaluations on plasma enzyme levels. All studies are in progress and final results are not yet available.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BIOASSAY SYSTEMS CORPORATION
Woburn, Massachusetts 01801
(NIH-N01-ES-28004-03)

TITLE: Prechronic Study of 9-Aminoacridine

CONTRACTOR'S PROJECT DIRECTOR: Indu Muni, Ph.D.

PROJECT OFFICER (NIEHS): June K. Dunnick, Ph.D., Toxicologist,
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: August, 1983

CURRENT ANNUAL LEVEL: \$118,147 and funded in previous year

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. There is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory is in the process of conducting prechronic toxicity studies with 9-aminoacridine (CAS No. 134-50-9). These studies are performed in accordance with the NTP Master Agreement and involve dosing the animals by feed route of exposure; noting clinical signs, body weight and food consumption during the study; and performing a gross and microscopic evaluation of animal tissues at the conclusion of the study. This laboratory is also performing clinical chemistry evaluations on plasma enzyme levels. All studies are in progress and final results are not yet available.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

LITTON BIONETICS, INC.
Rockville Facility
Kensington, Maryland 20795
(NIH-N01-ES-28009-02)

TITLE: Carcinogen Bioassay of p-Nitrotoluene and 4-Chloro-2-nitroaniline

CONTRACTOR'S PROJECT DIRECTOR: Alan G. Manus, Ph.D.

PROJECT OFFICER (NIEHS): Richard D. Irwin, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September, 1982

CURRENT ANNUAL LEVEL: \$32,315

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

p-Nitrotoluene - Prechronic complete.

4-Chloro-2-nitroaniline - Prechronic complete.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

PUBLICATIONS

Melnick, R.L., Boorman, G.A., Haseman, J.K., Montali, R.J. and Huff, J.E.: Urolithiasis and bladder carcinogenicity of melamine in rodents. Toxicol. Appl. Pharmacol., 72: 292-303, 1984.

LITTON BIONETICS, INC.
Rockville Facility
Kensington, Maryland 20795
(NIH-NO1-ES-28009-03)

TITLE: Carcinogen Bioassay of Vinylidene Fluoride

CONTRACTOR'S PROJECT DIRECTOR: Alan G. Manus, Ph.D.

PROJECT OFFICER (NIEHS): Richard D. Irwin, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: August, 1982

CURRENT ANNUAL LEVEL: \$24,317

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Vinylidene Fluoride - Prechronic complete.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

LITTON BIONETICS, INC.
Rockville Facility
Kensington, Maryland 20795
(NIH-N01-ES-28009-04)

TITLE: Carcinogen Bioassay of 2-Mercaptobenzimidazole and Isobutyraldehyde

CONTRACTOR'S PROJECT DIRECTOR: Alan Manus, Ph.D.

PROJECT OFFICER (NIEHS): Richard Irwin, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September, 1982

CURRENT ANNUAL LEVEL: \$ 00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Isobutyraldehyde - Prechronic complete.

2-Mercaptobenzimidazole - Prechronic complete.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 28026
(NIH-N01-ES-28011-02)

TITLE: Toxicity Testing of d-Alpha Tocopheryl Acetate

CONTRACTOR'S PROJECT DIRECTOR: Lois T. Mulligan, Ph.D.

PROJECT OFFICER (NIEHS): Kame1 M. Abdo, Ph.D., Chemist
Carcinogenicity and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: December, 1983

CURRENT ANNUAL LEVEL: \$7,007

PROJECT DESCRIPTION

OBJECTIVES: d-alpha-Tocopheryl Acetate (vitamin E) is being consumed in excessive quantities (up to 100 times the recommended daily allowance) by health conscious individuals and food faddists without regard to the consequence of such excesses. Numerous deleterious effects have been reported following excess intake of this vitamin in both humans and experimental animals. Example of such effects included muscle weakness, creatinurea and elevated serum creatine, cholesterol and triglycerides in humans, and altered hematological profile and increased requirements for vitamin D and K. Because of these findings and the potential for and actual use of very high amounts of this vitamin a carefully controlled and detailed study of vitamin E was conducted in two strains of rats (Fischer 344 and Sprague-Dawley) to provide detailed information about the toxic effects of this compound.

METHODS EMPLOYED: Subchronic (13-week) investigations conducted under the contract included the extensive use of clinical chemistry evaluation, hematology, sperm morphology and vaginal cytology evaluation, and histopathology. The studies were conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life data were collected by computer using the NTP Toxicology Data Management System. Other data including gross observations at necropsy and histopathology were collected using manual methods.

MAJOR FINDINGS: The laboratory toxicology and draft histopathology reports were received by NTP for quality assurance and pathology working group evaluation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-28013-03)

TITLE: Repeated Dose and Subchronic Toxicological Evaluations of Theophylline in Rats and Mice

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: October 1, 1983

CURRENT ANNUAL LEVEL: \$92,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Theophylline - Prechronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-28013-04)

TITLE: Repeated-Dose and Subchronic Toxicological Evaluations of
1,2-Dihydro-2,2,4-Trimethylquinoline in Rats and Mice

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: October 7, 1983

CURRENT ANNUAL LEVEL: \$264,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Prechronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SRI INTERNATIONAL
Menlo Park, California 94025
(NIH-N01-ES-28015)

TITLE: Bioassay Testing of Barium Chloride and Tetrahydrocannabinol

CONTRACTOR'S PROJECT DIRECTOR: Ted A. Jorgenson

PROJECT OFFICER (NIEHS): Douglas W. Bristol, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1982

CURRENT ANNUAL LEVEL: \$100,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Barium Chloride - Prechronic reports submitted and accepted following review by the PWG. Work on this project is complete.

Tetrahydrocannabinol - Prechronic report submitted for rats; mouse report near completion; awaiting PWG review.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BIOASSAY SYSTEMS CORPORATION
Woburn, Massachusetts 01801
(NIH-N01-ES-28022)

TITLE: Chronic Study of Hydroquinone

CONTRACTOR'S PROJECT DIRECTOR: Indu Muni, Ph.D.

PROJECT OFFICER (NIEHS): June K. Dunnick, Ph.D., Toxicologist,
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: April, 1982

CURRENT ANNUAL LEVEL: Funded in previous year

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. There is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory is in the process of conducting chronic toxicity studies with hydroquinone. These studies are performed in accordance with the NTP Master Agreement and involve dosing the animals by the gavage route of exposure; noting clinical signs, body weight and food consumption during the study; and performing a gross and microscopic evaluation of animal tissues at the conclusion of the study. This laboratory is also performing clinical chemistry evaluations on plasma enzyme levels. All studies are in progress and final results are not yet available.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

HAZLETON LABORATORIES AMERICA, INC.
Vienna, Virginia 22180
(NIH-N01-ES-28023-04)

TITLE: Chronic Toxicity and Carcinogenicity Studies of C.I. Direct Blue 15, C.I. Acid Red 114, 3,3'-Dimethylbenzidine and 3,3'-Dimethoxybenzidine in Fischer 344 Rats

CONTRACTOR'S PROJECT DIRECTOR: Borge Ulland, Ph.D.

PROJECT OFFICER (NIEHS): John H. Mennear, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: \$188,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

N01-ES-28023-04 - Chronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-28024)

TITLE: Bioassay Testing for Disperse Blue 1, H.C. Blue 2, Nitrofurantoin, Rhodamine 6G, H.C. Red 3, Chlorowax 500C, Acid Orange 3, Dichlorvos, Chlorowax 40, Roxarsone, and Pentachloroanisole

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: May 31, 1982

CURRENT ANNUAL LEVEL: \$300,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

C.I. Disperse Blue 1 - Technical Report Peer Reviewed (3/85).

H.C. Blue 2 - Technical Report Peer Reviewed (7/84).

Nitrofurantoin - Chronic histopathology completed; PWG pending.

Rhodamine 6G - Chronic histopathology in progress.

H.C. Red 3 - Technical Report Peer Reviewed (3/85).

Chlorowax 500C - Technical Report being drafted.

Acid Orange 3 - Rat chronic histopathology PWG approved (3/85); mouse
chronic histopathology completed (PWG pending).

Dichlorvos - Chronic histopathology completed; PWG pending.

Chlorowax 40 - Technical Report being drafted.

Roxarsone - Chronic histopathology in progress.

Pentachloroanisole - Chronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

PAPANICOLAOU CANCER RESEARCH INSTITUTE
Miami, Florida 33136
(NIH-N01-ES-28025)

TITLE: Bioassay Testing of Trichloroethylene, Isophorone, Glycidol, 2,2-Bis-(bromomethyl)1,3-propanediol, and 2,3-Dibromo-1-propanol.

CONTRACTOR'S PROJECT DIRECTOR: Fred G. Bock, Ph.D.

PROJECT OFFICER (NIEHS): Michael P. Dieter, Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: July 15, 1982

CURRENT ANNUAL LEVEL: \$ 00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Chronic testing for trichloroethylene and isophorone are completed; testing for glycidol is awaiting verification of pathology findings. Subchronic testing for 2,2-Bis(bromomethyl)1,3-propanediol and 2,3-dibromo-1-propanol are awaiting pathology completion.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20816
(NIH-N01-ES-28026)

TITLE: Bioassay Testing of D-Limonene, Succinic Anhydride, Alpha-Methylbenzyl Alcohol, Benzyl Alcohol and Methyl Carbamate.

CONTRACTOR'S PROJECT DIRECTOR: Lois T. Mulligan, Ph.D.

PROJECT OFFICER (NIEHS): Kamal M. Abdo, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: August 2, 1982

CURRENT ANNUAL LEVEL: \$3,200

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory materials and methods reports and draft pathology narrative for the studies with the above chemicals were received by NTP for quality assurance and pathology working group evaluation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

LITTON BIONETICS, INC.
Rockville Facility
Kensington, MD 20795
(NIH-N01-ES-38040)

TITLE: Bioassay Testing of Various Chemicals (Dimethylmethylphosphonate and Dimethylvinylchloride)

CONTRACTOR'S PROJECT DIRECTOR: Alan G. Manus, Ph.D.

PROJECT OFFICER (NIEHS): Richard D. Irwin, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: March, 1983

CURRENT ANNUAL LEVEL: \$ 00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Dimethylmethylphosphonate - Chronic sacrifice completed; pending PWG.

Dimethylvinylchloride - Technical Report in preparation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

PUBLICATIONS

Kluwe, W.M., Parker, G.A. and Manus, A.G.: Chronic toxicity of diallylphthalate in mice. Toxicol. Lett., (in press), 1984.

PHYSIOLOGICAL RESEARCH LABORATORIES
Division of Medtronic, Inc.
Minneapolis, Minnesota 55433
(NIH-N01-ES-38041)

TITLE: Bioassay Testing of 2-Amino-4-nitrophenol, 2-Amino-5-nitrophenol, Ephedrine Sulfate, Erythromycin Stearate, Hexylresorcinol, Mercaptobenzothiazole, Methyldopa, Nalidixic Acid, Nitrofurazone, Oxytetracycline Hydrochloride, Phenylephrine Hydrochloride, and Tetracycline Hydrochloride.

CONTRACTOR'S PROJECT DIRECTOR: Morris J. Cowan, Jr.

PROJECT OFFICER (NIEHS): Ronald L. Melnick, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: March 1, 1983

CURRENT ANNUAL LEVEL: \$225,665

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The two-year treatments with all of the 12 chemicals have been completed. All studies are in various stages of histopathologic evaluation. These evaluations are expected to be completed in FY85. All findings from these studies will be reported after audits and internal and peer reviews of the data have been performed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

MIDWEST RESEARCH INSTITUTE
Kansas City, Missouri 64110
(NIH-N01-ES-38042)

TITLE: Chronic Toxicity Testing of Vinyl Toluene and Tetranitromethane

CONTRACTOR'S PROJECT DIRECTOR: James M. Cholakis, Ph.D.

PROJECT OFFICER (NIEHS): Joseph H. Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$202,912

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Chronic inhalation studies with vinyl toluene and tetranitromethane were completed and reported.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SPRINGBORN INSTITUTE FOR BIORESEARCH, INC.
Spencerville, Ohio 45887
(NIH-N01-ES-38043)

TITLE: Long-term Rodent Carcinogenicity Bioassay on Ampicillin Trihydrate, Benzofuran, N,N-dimethylaniline and Penicillin VK.

CONTRACTOR'S PROJECT DIRECTOR: Richard A. Hiles, Ph.D.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: Phased over from Tracor Jitco 9-30-83

CURRENT ANNUAL LEVEL: \$166,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: All laboratory work will be completed this year. The contract expiration date is July 31, 1985.

Ampicillin Trihydrate - Final chronic report was submitted to NTP. Technical Report is in preparation.

Penicillin VK - Final chronic report was submitted to NTP. Technical Report is in preparation.

Benzofuran - Pathology is in progress.

N,N-dimethylaniline - Pathology is in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

A.D. LITTLE, INCORPORATED - Cambridge, Massachusetts
(NIH-N01-ES-38050)

TITLE: National Toxicology Program Health and Safety Support

CONTRACTOR'S PROJECT DIRECTOR: R. Scott Stricoff

PROJECT OFFICER (NIEHS): D.B. Walters, Ph.D., Head, Chemical Health and Safety,
Collaborative Resources Group, Carcinogenesis and
Toxicology Evaluation Branch, TRTP

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$526,746

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this Contract is to assist the National Toxicology Program in the evaluation of health and safety practices at its contract laboratories. Assistance provided includes: basic industrial hygiene as well as specialized experience in analytical chemistry, engineering control, personal protective equipment evaluation, human factors evaluation and design of sampling strategies.

METHODS EMPLOYED: The Contractor furnishes services, qualified personnel, material, equipment and facilities as needed to evaluate, survey and assist the NTP in the following areas:

1. Propose and develop alternate work practices and/or engineering controls for use in eliminating or greatly lessening the risk for potentially hazardous situations encountered in facilities used for or in support of toxicology testing. This task may include developmental research on alternate work practices, areas, equipment or facilities.
2. Conduct baseline health and safety and follow-up site visits at NTP contract laboratories conducting lifetime rodent toxicology bioassays, cellular and genetic toxicology, systemic toxicology, chemistry and repository functions.
3. Provide information and data which will enable the project officer to prepare final versions of chemical specific health and safety guidelines, standards, documents and safety plans for work with NTP test chemicals. Included in this task is the development of predetermined NTP training needs and materials.
4. Determine performance characteristics and suggest design changes where appropriate, of ventilation systems, incinerators, work stations, protective equipment.
5. Develop, design and apply industrial hygiene, chemistry and pollution monitoring programs as directed by the Project Officer. Such monitoring may examine the spread of test chemicals within the facility as a result of NTP activities. Sampling methods shall be capable of quantifying trace levels of test chemicals which correspond with the toxicological risk of the chemical.

MAJOR FINDINGS AND PROPOSED COURSE: Program accomplishments for FY 1985 include the establishment of a basic level of safety awareness at the NTP contract laboratories and continuation of the site visit at a maintenance level. Approximately 25 contract laboratories were visited during FY 1985 as part of a scheduled maintenance program, along with visits to several laboratories which either lacked strong safety programs or which have had significant facility modifications. Approximately 50 Health and Safety Packages were prepared in an ongoing effort to provide chemical specific information during the protocol design and testing stages. Accomplishments for the technical assessment and control program area for FY 1985 include: 1) the design of a dosing work station to minimize exposure during gavage, skin paint and dosed feed filling operations; 2) the evaluation of a tissue trimming work station; 3) the design and evaluation of a vented balance enclosure; 4) the glove permeation and tactility evaluation of several glove types for use during operations involving formalin; and 5) the review and evaluation of fire and explosion protection during inhalation testing.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The National Toxicology Program has a national mandate to determine the toxicologic potential of environmental chemicals. The objective is primarily attained by testing various chemicals in both long term animal studies and short-term tests at contract laboratories located throughout the U.S. and abroad. The maintenance of an effective Health and Safety Program is an essential part in maintaining the quality of the Program. Also, in order to minimize liability, NTP must monitor and supervise its contractor's procedures to assure that the hazards of handling test substances are controlled.

PUBLICATIONS

Walters, D. B., Prokopetz, A. T. and Stricoff, R. S.: Safety Recommendations for Handling Polynuclear Aromatic Hydrocarbons in PAH: Human Exposure and Health Effects, Battelle Press, Columbus, 1985.

BATTELLE MEMORIAL INSTITUTE
BATTELLE PACIFIC NORTHWEST LABORATORIES
Richland, Washington 99352
(NIH-N01-ES-38061)

TITLE: Chemical Testing of Various Chemicals

CONTRACTOR'S PROJECT DIRECTOR: Harvey A. Ragan, D.V.M.

PROJECT OFFICER (NIEHS): Joseph H. Roycroft, Ph.D., Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: March 15, 1983

CURRENT ANNUAL LEVEL: \$2,409,413

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: During 1984 6 chronic inhalation studies were completed (ethyl bromide, ethyl chloride, allyl glycidyl ether, -chloroacetophenone, epinephrine-HCl, and o-chlorobenzalmalonitrile). Final chronic reports for 4 inhalation studies (ethylene oxide, methylene chloride, tetrachloroethylene and methyl methacrylate) were submitted.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-38068)

TITLE: "Chemical Testing of: N-Phenyl- β -Naphthylamine, 2,4-Dichlorophenol, Sodium Fluoride, Pentachlorophenol (Technical Grade), Pentachlorophenol (Dowicide EC-7), Rotenone, and Chorpheniramine Maleate".

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: November 1, 1982

CURRENT ANNUAL FUNDING: \$278,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: All laboratory work will be completed this year. The contract expiration date is July 31, 1985.

N-Phenyl- β -Naphthylamine - Chronic study pathology was completed and the laboratory final report has been submitted to the NTP.

2,4-Dichlorophenol - Chronic study was completed in 1984 and the final laboratory report was submitted to the NTP. The technical report is in preparation.

Sodium Fluoride - The pathology was completed in 1984 and the final laboratory report has been submitted to the NTP.

Pentachlorophenol (technical grade) - The pathology was completed in September 1984 and the laboratory final chronic report has been submitted to the NTP.

Pentachlorophenol (Dowicide EC-7) - The pathology is completed and the laboratory final chronic report has been submitted to the NTP.

Rotenone - Pathology was completed in July 1984 and the laboratory final chronic report has been submitted to the NTP.

Chlorpheniramine Maleate - Pathology was completed in May 1984 and the final chronic report has been submitted to the NTP.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SRI INTERNATIONAL
Menlo Park, California 94025
(NIH-N01-ES-38069)

TITLE: Bioassay Testing of Diphenhydramine Hydrochloride, Furosemide, Hydrochlorothiazide, and 8-Methoxypsoralen.

CONTRACTOR'S PROJECT DIRECTOR: Ted A. Jorgenson

PROJECT OFFICER (NIEHS): Douglas W. Bristol, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: January 1, 1983

CURRENT ANNUAL LEVEL: \$266,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Chronic studies complete on all four compounds. Final report preparation proceeding for diphenhydramine hydrochloride, furosemide and hydrochlorothiazide; pathology in progress for 8-methoxypsoralen.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans.

PUBLICATIONS

J.K. Dunnick, W.E. Davis, T.A. Jorgenson, V.J. Rosen, and E.E. McConnell:
Subchronic Toxicity of Orally Administered 8-Methoxypsoralen in Fischer 344
Rats. J. Natl. Cancer Institute, (in press) 1984.

LITTON BIONETICS, INC.
Rockville Facility
Kensington, Maryland 20795
(NIH-N01-ES-45036)

TITLE: Toxicity Tests of Butyl Benzyl Phthalate, Diethylphthalate and Dimethylphthalate

CONTRACTOR'S PROJECT OFFICER: Allan G. Manus, Ph.D.

PROJECT OFFICER (NIEHS): Richard D. Irwin, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September, 1982

CURRENT ANNUAL LEVEL: \$ 00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Diethylphthalate - 28-Day repeated dose study completed; chronic dermal study in progress.

Butyl Benzyl Phthalate - 28-Day repeated dose study completed; 180-day prechronic study and modified mating trial in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-45037)

TITLE: Chronic Toxicity/Carcinogenicity Study on Chloramine in Rats and Mice

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1984

CURRENT ANNUAL LEVEL: \$620,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Chloramine - Chronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazards to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-45038)

TITLE: Toxicity and Carcinogenicity Tests of p-Nitroaniline and o-Nitroanisole

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1984

CURRENT ANNUAL LEVEL: \$343,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

p-Nitroaniline - Chronic studies in progress.

o-Nitroanisole - Chronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BIOASSAY SYSTEMS CORPORATION
Woburn, Massachusetts 01801
(NIH-N01-ES-45039)

TITLE: Chronic Study of Coumarin and Dihydrocoumarin

CONTRACTOR'S PROJECT DIRECTOR: Indu Muni, Ph.D.

PROJECT OFFICER (NIEHS): June K. Dunnick, Ph.D., Toxicologist,
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: July, 1984

CURRENT ANNUAL LEVEL: \$76,970 and funded in previous year

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. There is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory is in the process of conducting prechronic toxicity studies 2,2-Bis(bromomethyl)-1,3-propanediol (CAS. No. 3296-90-0). These studies are performed in accordance with the NTP Master Agreement and involve dosing the animals by the feed route of exposure; noting clinical signs, body weight and food consumption during the study; and performing a gross and microscopic evaluation of animal tissues at the conclusion of the study. This laboratory is also performing clinical chemistry evaluations on plasma enzyme levels. All studies are in progress and final results are not yet available.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

LITTON BIONETICS, INC.
Rockville Facility
Kensington, Maryland 20795
(NIH-N01-ES-45040)

TITLE: Chronic Toxicity and Carcinogenicity Test of p-Nitrophenol

CONTRACTOR'S PROJECT OFFICER: Allan G. Manus, Ph.D.

PROJECT OFFICER (NIEHS): Richard D. Irwin, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1984

CURRENT ANNUAL LEVEL: \$53,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

p-Nitrophenol - Chronic dermal study in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-45041)

TITLE: "Toxicity and Carcinogenicity Studies of Manganese Sulfate and Triamterene"

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1984

CURRENT ANNUAL FUNDING: \$44,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

N01-ES-45041:

Manganese Sulfate - This chronic feed study in F344 rats and B6C3F₁ mice includes 3 dose levels plus untreated controls. Interim evaluations are scheduled at 9 and 15 months. In addition to pathology, reproductive effects will be assessed after 9 months and clinical chemistries, tissue manganese, iron, copper and zinc will be assessed at 9 and 15 months.

Triamterene - Administered by 3 dose levels (plus untreated controls) to F344 rats and B6C3F₁ mice for 103 weeks. A 15 month interim evaluation will be performed and clinical chemistries and pathology assessment will be made. Separate groups of animals were included to measure reproductive effects at 13 weeks.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-45042)

TITLE: Toxicity and Carcinogenicity Tests of o-Benzyl-p-chlorophenol

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1984

CURRENT ANNUAL FUNDING: \$41,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

o-Benzyl-p-chlorophenol - Exposure of F344 rats and B6CF₁ mice to 3 dose levels of this chemical by gavage plus vehicle controls will continue for 103 weeks. Rats will be tested for urine concentrating ability, porphyrin, and clinical chemistries at intervals throughout the study. Reproductive effects will be determined after 3 months. Interim sacrifices are scheduled for 9 and 15 months of dosing.

Mouse skin initiation/promotion studies are also underway with o-benzyl-p-chlorophenol.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

RADIAN CORPORATION - AUSTIN, TEXAS
(NO1-ES-45043)

TITLE: Chemistry Support for Toxicity Testing at NIEHS

CONTRACTOR'S PROJECT DIRECTOR: R. Moseman, Ph.D.

PROJECT OFFICERS (NTP): C.W. Jameson, Ph.D., Head, Collaborative Resources Group, and T.J. Goehl, Ph.D., Master Agreement Chemistry Leader, CRG, Carcinogenesis and Toxicology Evaluation Branch, TRTP

DATE CONTRACT INITIATED: September 24, 1984

CURRENT ANNUAL LEVEL: \$420,000

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this contract is to provide chemical procurement, analysis, storage, repackaging, and distribution services in support of the intramural TRTP research efforts at the NIEHS. The contractor serves as an analytical chemistry resource for the TRTP in-house research effort by 1) performing analysis of chemicals for identity, purity and stability; 2) devising procedures for preparation and analysis of test chemicals in dosage formulations, and 3) preparing and analyzing dose formulations for studies and providing these formulations to investigators at NIEHS on a routine basis. Other tasks also required include isolation and identification of impurities, tissue residue analyses and other associated analytical problems.

METHODS EMPLOYED: The contractor procures and receives chemicals which are to be investigated by various TRTP researchers at NIEHS. Data is generated on the identity and purity of each test material. In addition stability assays are performed to determine the long term stability of both the bulk chemical and chemical-vehicle mixtures. Methods are also developed for the assay of the test material in the vehicle used in the toxicity study. Dose formulations of the test material in the appropriate vehicle are then prepared and provided to the TRTP investigator on a routine basis. Other assays, such as tissue residue analysis and identification of minor impurities are performed as required.

MAJOR FINDINGS AND PROPOSED COURSE: For FY 85, 19 chemicals were procured and/or analyzed for study by the TRTP at the NIEHS. This work included routine dose mixing and analysis as well as tissue and body fluid analysis for selected test materials. In addition routine dose analysis was provided for two chemicals being studied in the NTP's Continuous Breeding Program. Referee analysis was provided for dose mixtures of two chemicals being studied in the NTP's Teratology Program. Future plans include continued support of the in-house research efforts of the TRTP.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The National Toxicology Program has a national mandate to determine the toxicologic potential of environmental chemicals. The object is primarily attained by the testing of various chemicals in both long term animal studies and short-term tests. However it is also necessary for the TRTP to perform in-house research to evaluate test methods and verify results reported elsewhere. The procurement and analysis of chemicals for this in-house research effort is one of the essential steps in the success of toxicity studies. Without this activity, no substantive animal or in vitro research could occur. The precise definition of the chemical nature of test compounds is one of the cornerstones in an effort to increase the accuracy and reliability of data obtained in toxicological research.

BIOASSAY SYSTEMS CORPORATION
Woburn, Massachusetts 01801
(NIH-N01-ES-45047)

TITLE: Prechronic Study of 2,2-Bis(Bromomethyl)-1,3-propanediol

CONTRACTOR'S PROJECT DIRECTOR: Indu Muni, Ph.D.

PROJECT OFFICER (NIEHS): June K. Dunnick, Ph.D., Toxicologist,
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September, 1984

CURRENT ANNUAL LEVEL: Funded in previous year

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. There is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory is in the process of conducting prechronic carcinogenicity studies with 2,2-Bis(Bromomethyl)-1,3-propanediol. These studies are performed in accordance with the NTP Master Agreement and involve dosing the animals by the feed route of exposure; noting clinical signs, body weight and food consumption during the study; and performing a gross and microscopic evaluation of animal tissues at the conclusion of the study. This laboratory is also performing clinical chemistry evaluations on plasma enzyme levels. All studies are in progress and final results are not yet available.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-45050)

TITLE: Toxicity and Carcinogenicity Tests of Tricresyl Phosphate

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL FUNDING: \$437,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Tricresyl Phosphate - A 13 week feeding study was completed and evaluated for changes in hematology, grip strength, serum cholinesterase and reproduction. Results from the subchronic are used to determine the chronic doses. The chronic feeding study includes exposure to chemical at 3 dose levels plus untreated controls to F344 rats and B6C3F₁ mice. Interim evaluation will be made at 6, 12 and 15 months of dosing and hematology, serum cholinesterase, grip strength and neurohistopathology will be measured.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

RADIAN CORPORATION - AUSTIN, TEXAS
(N01-ES-45059)

TITLE: National Toxicology Program Chemical Repository and Safety Support

CONTRACTOR's PROJECT DIRECTOR: L. H. Keith, Ph.D.

PROJECT OFFICERS (TRTP): D.B. Walters, Ph.D. and A.T. Prokopetz, Chemical Health and Safety, Carcinogenesis and Toxicology Evaluation Branch, TRTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL LEVEL: \$1,427,828

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this contract is to establish a repository with initial capacity of 2,000 unique chemicals and final capacity of approximately 4,000 unique chemicals for toxicity screening in the National Toxicology Program. This contract provides a mechanism whereby approximately 350 test chemicals per year can be located, procured, analyzed for physical properties, stored and distributed in a manner which guarantees their safe handling, chemical integrity and maintains confidentiality of identity when necessary.

METHODS EMPLOYED: The repository receives a listing of chemicals which are to be tested either blind or as knowns by laboratories under contract. Concurrently with location and acquisition of these chemicals, the repository searches through on-line computer data bases to produce chemical specific handling documents both for day-to-day safe handling of the compounds as well as for emergency situations. The repository also determines the physical properties of those chemicals for which flash point, density and vapor pressure information is unavailable in the literature. Tracking and monitoring of repository functions are accomplished by a computerized database management system. This system generates randomized codes for the various aliquots which are to be tested blind and then produces a file containing the chemical, physical and toxicological properties for each repository aliquots. The compounds are doubly contained and shipped according to DOT regulations by the most appropriate, safest and expedient possible route to the testing laboratory.

MAJOR FINDINGS AND PROPOSED COURSE: Combination of the two previous repositories, the Carcinogenesis Bioassay Testing Repository (N01-ES-95649) and the National Toxicology Program Chemical Repository (N01-ES-15010), Was accomplished during the first year of this new contract. The computerized data management system maintains a tracking system to indicate the prior testing status of the combined repository chemicals. More than 1300 aliquots were sent to investigators for toxicity testing in FY 1985, either as coded blind samples or as uncoded shipments. Redesigned chemical specific documents are reviewed by a physician to ensure appropriate emergency response for these potentially toxic test compounds. Dust explosion and lower explosive limit determinations were initiated to assist personnel in the design of inhalation testing protocols and to ensure safe handling of potentially explosive test compounds. Glove permeation data on non-volatile test compounds are being determined by means of a

discrete sampling procedure. This information is utilized by the various branches of TRTP involved in in vivo testing. During FY 1986 the Chemical Repository Program will continue to provide assistance to the TRTP. Increased overseas shipments are anticipated as a cooperative effort with WHO and other branches of NIEHS is implemented.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The development of a comprehensive testing system requires a repository which can be computerized for effectiveness and efficiency and which is organized around a specially designed containment laboratory for handling hazardous materials. The laboratory also must provide for routine chemical assay as well as sophisticated, complete, chemical trace impurity analysis. These requirements are necessary to support in vitro and in vivo testing.

MIDWEST RESEARCH INSTITUTE - KANSAS CITY, MISSOURI
(N01-ES-45060)

TITLE: Chemical Services Support for the National Toxicology Program

CONTRACTOR'S PROJECT DIRECTOR: K.M. Stelting, Ph.D.

PROJECT OFFICERS (NTP): T.J. Goehl, Ph.D., Master Agreement Chemistry Leader
and C.W. Jameson, Ph.D., Head, Collaborative Resources
Group, Carcinogenesis and Toxicology Evaluation Branch,
TRTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL LEVEL: \$3,001,000

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this contract is to provide chemical procurement, analysis, storage, repackaging, and distribution services in support of the activities of the National Toxicology Program. The contractor serves as an analytical chemistry resource for the NTP by 1) performing analysis of chemicals for identity, purity and stability; 2) devising procedures for preparation and analysis of test chemicals in dosage formulations, 3) preparing protocols for use by testing laboratories for bulk chemical and dosage analyses, 4) acting as a referee laboratory, and 5) performing custom syntheses. Special tasks also include isolation and identification of impurities, tissue residue analyses and other associated analytical problems.

METHODS EMPLOYED: The contractor procures and receives chemicals which are to be tested by various Programs in the NTP including both contract and in-house laboratories. Data is generated on the identity and purity of each test material. In addition stability assays are performed to determine the long term stability of both the bulk chemical and chemical-vehicle mixtures. Also, methods are developed for the assay of the test material in the vehicle used in the toxicity test. Other assays, such as tissue residue analysis and identification of minor impurities, are performed as required.

MAJOR FINDINGS AND PROPOSED COURSE: For FY 85, 86 chemicals were procured and/or analyzed for toxicology and carcinogenesis testing. In addition, 11 chemicals were procured and/or analyzed for teratology studies. Analytical services were provided for the NTP's Continuous Breeding Program of the Reproductive Toxicology Section by procuring or synthesizing and analyzing 9 chemicals for study and providing routine analytical chemistry services for the contract laboratories of this program. Support for other NTP Programs, including immunology, chemical disposition and the in vivo rat liver model was also accomplished with the procurement and/or analysis of five chemicals. Work was also completed on routine dose mixing and analysis as well as tissue and body fluid residue analysis for chemicals being studied by various members of the TRTP intramural staff. Future plans include continued support of the above mentioned activities as well as support of any new NTP initiatives.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The National Toxicology Program has a national mandate to determine the toxicologic potential of environmental chemicals. The object is primarily attained by the testing of various chemicals in both long term animal studies and short-term tests. The procurement and analysis of chemicals is one of the essential steps in the success of toxicity studies. Without this activity, no substantive animal or in vitro testing could occur. The precise definition of the chemical nature of test compounds is one of the cornerstones in an effort to increase the accuracy and reliability of data obtained in toxicological research.

RESEARCH TRIANGLE INSTITUTE - RESEARCH TRIANGLE PARK, NORTH CAROLINA
(N01-ES-45061)

TITLE: Chemical Services Support for the National Toxicology Program

CONTRACTOR'S PROJECT DIRECTOR: M. Wall, Ph.D.

PROJECT OFFICERS (NTP): C.W. Jameson, Ph.D., Head, Collaborative Resources Group, and T.J. Goehl, Ph.D., Master Agreement Chemistry Leader, CRG, Carcinogenesis and Toxicology Evaluation Branch, TRTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL LEVEL: \$2,616,000

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this contract is to provide chemical procurement, analysis, storage, repackaging, and distribution services in support of the activities of the National Toxicology Program. The contractor serves as an analytical chemistry resource for the NTP by 1) performing analysis of chemicals for identity, purity and stability; 2) devising procedures for preparation and analysis of test chemicals in dosage formulations, 3) preparing protocols for use by testing laboratories for bulk chemical and dosage analyses, 4) acting as a referee laboratory, and 5) performing custom synthesis. Special tasks also required include isolation and identification of impurities, tissue residue analyses and other associated analytical problems.

METHODS EMPLOYED: The contractor procures and receives chemicals which are to be tested by various Programs in the NTP including both contract and in-house laboratories. Data is generated on the identity and purity of each test material. In addition stability assays are performed to determine the long term stability of both the bulk chemical and chemical-vehicle mixtures. Also, methods are developed for the assay of the test material in the vehicle used in the toxicity test. Other assays, such as tissue residue analysis and identification of minor impurities, are performed as required.

MAJOR FINDINGS AND PROPOSED COURSE: This contract was established in FY 85 to provide analytical chemistry services support mainly for the TRTP's Cellular and Genetic Toxicology Program. For FY 85, 248 chemicals were received and analyzed for purity and identity for the Cellular and Genetic Toxicology Program. In addition, three chemicals were procured and/or analyzed for teratology studies. Support for other NTP Programs, including immunology, and the in vivo rat liver model was also accomplished with the procurement and/or analysis of nine chemicals. Work was completed on routine dose formulation analysis as well as tissue and body fluid residue analysis for two chemicals being studied by various members of the TRTP intramural staff. Future plans include continued support of the above mentioned activities as well as expanded support for the NTP's Continuous Breeding Program of the Reproductive Toxicology Section and the Master Agreement Carcinogenesis Testing Program.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The National Toxicology Program has a national mandate to determine the toxicologic potential of environmental chemicals. The object is primarily attained by the testing of various chemicals in both long term animal studies and short-term tests. The procurement and analysis of chemicals is one of the essential steps in the success of toxicity studies. Without this activity, no substantive animal or in vitro testing could occur. The precise definition of the chemical nature of test compounds is one of the cornerstones in an effort to increase the accuracy and reliability of data obtained in toxicological research.

LITTON BIONETICS, INC.
Rockville Facility
Kensington, Maryland 20795
(NIH-N01-ES-45064)

TITLE: Toxicity Tests of Crotonaldehyde and Butyraldehyde

CONTRACTOR'S PROJECT OFFICER: Allan G. Manus, Ph.D.

PROJECT OFFICER (NIEHS): Richard D. Irwin, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL LEVEL: \$49,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Crotonaldehyde - 14-Day repeated dose gavage study completed.

Butyraldehyde - 14-Day repeated dose gavage study completed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-NO1-ES-45066)

TITLE: Toxicity Studies of Benzethonium Chloride

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL FUNDING: \$24,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Benzethonium chloride - Two-week and 90-day exposures to F344 rats and B6C3F₁ mice by skin paint are underway. After 90 days, reproductive effects will be assessed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-45068)

TITLE: Toxicity Studies of Triethenolamine

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL FUNDING: \$257,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Triethanolamine - Using both F344 rats and B6C3F₁ mice, this chemical was given for two weeks to separate groups of each species by dosed water, inhalation, and dermal routes of exposure to determine the most appropriate route of exposure for a 90 day study. The 90 day study includes periodic sampling for analysis of hematology, urinalysis and selected clinical chemistries. Mating trials are planned.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

LITTON BIONETICS, INC.
Kensington, Maryland 20895
(NIH-N01-ES-48051)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Allan G. Manus, D.V.M., M.S.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Master Agreement order awards indicated below have been made under the current Master Agreement. The respective contract should be referred to for details of the current status.

<u>Contract No.</u>	<u>Study</u>	<u>Awarded Amount</u>
N01-ES-45036	Butyl benzyl phthalate Diethyl phthalate Dimethyl & diethyl phthalate	\$1,083,995
N01-ES-45040	p-Nitrophenol	\$ 387,716
N01-ES-45064	Crotonaldehyde Butyraldehyde	\$ 900,170
N01-ES-55101	Tetrahydrofuran	\$ 217,293

This Master Agreement is administratively active for the above listed contracts but has been superseded by NIH-N01-ES-55088 for future Master Agreement Order competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement will provide information about the toxicity of chemicals in experimental animals, which will be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BIOASSAY SYSTEMS CORPORATION
Woburn, Massachusetts 01801
(NIH-N01-ES-48052)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Indu A. Muni, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Master Agreement Order awards listed below have made under this Master Agreement and should be referred to for the current status of the studies.

<u>Contract No.</u>	<u>Study</u>	<u>Awarded Amount</u>
N01-ES-45039	Coumarin 3,4-Dihydrocoumarin	\$2,668,609
N01-ES-45047	2,2-Bis-(bromomethyl)-1, 3-propanediol	\$1,681,953

This Master Agreement is administratively active for the above listed awards but has been superseded by NIH-N01-ES-55085 for future Master Agreement competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement will provide information about the toxicity of chemicals in experimental animals, which will be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

HAZLETON LABORATORIES AMERICA, INC.
Vienna, Virginia 22180
(NIH-N01-ES-48053)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Borge M. Ulland, D.V.M.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: This contractor submitted no proposals under the subject Master Agreement thus no awards were made.

This Master Agreement has been superseded by NIH-N01-ES-55087 for future Master Agreement Order competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20816
(NIH-N01-ES-48054)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Louis T. Mulligan, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Master Agreement order awards indicated below have been made under the current Master Agreement. The respective contract should be referred to for details of the current status.

<u>Contract No.</u>	<u>Study</u>	<u>Awarded Amount</u>
N01-ES-55077	Hexachloro-1-3, Butadiene	\$ 85,300
N01-ES-55096	CI Direct Blue 218	\$911,164

This Master Agreement is administratively active for the above listed contracts but has been superseded by NIH-N01-ES-55089 for future Master Agreement Order competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement will be provide information about the toxicity of chemicals in experimental animals, which will be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

EG&G MASON RESEARCH INSTITUTE
Worcester, Massachusetts 01608
(NIH-N01-ES-48055)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Herman Lilja, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Master Agreement order awards indicated below have been awarded under this Master Agreement. The respective contract should be referred to for details on the current status.

<u>Contract No.</u>	<u>Study</u>	<u>Awarded Amount</u>
N01-ES-45055	Salicylazosulfapyridine	\$ 170,628
N01-ES-45048	1,2,3-Trichloropropane	\$ 739,965
N01-ES-45069	Promethazine hydrochloride	\$ 751,791
N01-ES-45051	2-Hydroxy-4-methoxy benzophenone	\$ 361,069
N01-ES-55075	Tetrachlorophthalic Acid	\$ 152,906

This Master Agreement is administratively active for the above listed contracts but has been superseded by NIH-N01-ES-48055 for future Master Agreement Order competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement will provide information about the toxicity of chemicals in experimental animals, which will be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
Battelle Columbus Laboratory
Columbus, Ohio 43201
(NIH-N01-ES-48056)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Master Agreement orders indicated below have been awarded under this Master Agreement. The respective contract should be referred to for specific information to date.

<u>Contract No.</u>	<u>Study</u>	<u>Awarded Amount</u>
N01-ES-45068	Triethanolamine	\$ 373,882
N01-ES-45050	Tricresyl phosphate	\$1,249,725
N01-ES-45041	Manganese sulfate Triamterene	\$1,588,262
N01-ES-45066	Benzethonium chloride	\$ 148,546
N01-ES-45042	O-Benzyl-p-chlorophenol	\$1,805,564
N01-ES-55072	Initiation/Promotion MNNG, Benzoyl peroxide, TCPA, DMBA	\$1,810,447
N01-ES-55076	1,6-Hexanediamine	\$ 492,757
N01-ES-55079	t-Butyl-Perbenzoate	\$ 219,998

This Master Agreement is administratively active for the above listed awards but has been superseded by NIH-N01-ES-55083 for future Master Agreement competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255-5305
(NIH-N01-ES-48057)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Joe David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: Master Agreement Awards indicated below have been made under this Master Agreement. The appropriate contract should be referred to for details on the current status.

<u>Contract No.</u>	<u>Study</u>	<u>Awarded Amount</u>
N01-ES-45037	Chloramine	\$1,639,646
N01-ES-45038	o-Nitroanisole p-Nitroaniline	\$1,178,363
N01-ES-55081	Scopolamine H Br.	\$ 206,420
N01-ES-55082	Benzyl Acetate	\$ 917,682

This Master Agreement will remain administratively active for all Master Agreement Order awards made under it but has been superseded by NIH-N01-ES-48057 for future Master Agreement Order competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement will provide information about the toxicity of chemicals in experimental animals, which will be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
Battelle Pacific Northwest Laboratory
Richland, Washington 99352
(NIH-N01-ES-48058)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Dr. H.A. Ragan

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1984

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: The following Master Agreement Order award has been made under this Master Agreement and should be referred to for specific details of the current status.

<u>Contract No.</u>	<u>Study</u>	<u>Awarded Amount</u>
N01-ES-55103	Tetrafluroethylene	\$ 620,781

This Master Agreement will remain administratively active for all Master Agreement order awards made under it but has been superseded by NIH-N01-ES-55084 for future Master Agreement competitions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement will provide information about the toxicity of chemicals in experimental animals, which will be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-55072)

TITLE: Dermal Initiation/Promotion Studies: Comparison of Three Mouse Strains

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: December 31, 1984

CURRENT ANNUAL FUNDING: \$832,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Dermal Initiation/Promotion Comparison Mouse Studies - The mouse skin initiation/promotion model is being used to compare the sensitivity of three mouse strains: the B6C3F₁, the Swiss CD-1 and the Sencar. Initiations being used are DMBA and MNNG; promoters with each initiation are TPA and Benzoyl Peroxide.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 55076
(NIH-N01-ES-55076)

TITLE: Toxicity Tests of 1,6-Hexanediamine

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: December 31, 1984

CURRENT ANNUAL FUNDING: \$493,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

1,6-Hexanediamine - F344 rats and B6C3F₁ mice were exposed to 5 doses of chemical for 14 days by dosed water. Results will be used to set doses and identify target tissues for the 90 day studies to follow.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-55079)

TITLE: Toxicity Tests of t-Butyl-perbenzoate

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: December 31, 1984

CURRENT ANNUAL FUNDING: \$176,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

t-Butyl-perbenzoate - Prechronic gavage studies are underway to examine the effects of t-Butyl-perbenzoate, and two potential metabolites, t-butanol and benzoic acid.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-55081)

TITLE: Toxicity Test of Scopolamine Hydrobromide

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February 1, 1985

CURRENT ANNUAL LEVEL: \$206,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Scopolamine Hydrobromide - Prechronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-55082)

TITLE: Toxicity and Carcinogenicity Studies of Benzyl Acetate

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February 1, 1985

CURRENT ANNUAL LEVEL: \$238,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Benzyl Acetate - Subchronic studies in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
Battelle Columbus Laboratory
Columbus, Ohio 43201
(NIH-N01-ES-55083)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: No awards have been made under this Master Agreement.

All Master Agreement Awardees will be reviewed biannually to determine if the awardee is still qualified to compete for Master Agreement Orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
Battelle Pacific Northwest Laboratory
Richland, Washington 99352
(NIH-N01-ES-55084)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Dr. H.A. Ragan

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: No awards have been made under this Master Agreement.

All Master Agreement awards will be reviewed biannually to determine if they are still qualified to compete for Master Agreement orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BIOASSAY SYSTEMS CORPORATION
Woburn, Massachusetts 01801
(NIH-N01-ES-55085)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Indu A. Muni, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: No awards have been made under this Master Agreement.

All Master Agreement Awardees will be reviewed biannually to determine if the awardee is still qualified to compete for Master Agreement Orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

EG&G MASON RESEARCH INSTITUTE
Worcester, Massachusetts 01608
(NIH-N01-ES-55086)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Herman Lilja, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: No awards have been made under this Master Agreement.

All Master Agreement Awardees will be reviewed biannually to determine if the awardee is still qualified to compete for Master Agreement Orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

HAZLETON LABORATORIES AMERICA, INC.
Vienna, Virginia 22180
(NIH-N01-ES-55087)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Borge M. Ulland, D.V.M.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: No awards have been made under this Master Agreement.

All Master Agreement Awardees will be reviewed biannually to determine if the awardee is still qualified to compete for Master Agreement Orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

LITTON BIONETICS, INC.
Kensington, Maryland 20895
(NIH-N01-ES-55088)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Allan G. Manus, D.V.M., M.S.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: There have been no awards made under this Master Agreement.

All Master Agreement Awardees will be reviewed biannually to determine if the awardee is still qualified to compete for Master Agreement Orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20816
(NIH-N01-ES-55089)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Louis T. Mulligan, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: No awards have been made under this Master Agreement.

All Master Agreement Awardees will be reviewed biannually to determine if the awardee is still qualified to compete for Master Agreement Orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255-5305
(NIH-N01-ES-55090)

TITLE: Master Agreement Award for Toxicity and Carcinogenicity Studies in Laboratory Animals

CONTRACTOR'S PROJECT DIRECTOR: Joe David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Mina Lee Vernon, Ph.D., Head, Collaborative Services, Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: February, 1985

CURRENT ANNUAL LEVEL: \$00.00

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials utilizing the Master Agreement contract mechanism to prescreen laboratories and thus speed up the award of master agreement orders for the conduct of specific studies. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F1 mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to group of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE: No awards have been made under this Master Agreement.

All Master Agreement awards will be reviewed biannually to determine if they are still qualified to compete for Master Agreement orders.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of any studies conducted under this Master Agreement would provide information about the toxicity of chemicals in experimental animals, which would be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-82151)

TITLE: Comparative Carcinogenicity and Toxicity Studies of Selected Environmental Chemicals in Laboratory Animals Exposed During Pre- and Postnatal Life

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): Rajendra S. Chhabra, Ph.D., Supv. Pharmacologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1978

CURRENT ANNUAL LEVEL: \$949,974

PROJECT DESCRIPTION

OBJECTIVES: The main objective of this study is to determine if there is increased sensitivity of laboratory animals to potential carcinogenic and toxicologic effects of selected environmental chemicals exposed at various dose levels during their in utero development plus postnatal life of two years as compared to the animals exposed only during post-weaning time of two years. This objective will be achieved by (1) studying the carcinogenic potential of selected environmental chemicals exposed to the laboratory animals during their in utero development and weaning period (by treating their mothers with the chemical until weaning) followed by life time exposure (2 years) and comparing it with the effects observed in parallel groups of animals (derived from untreated mothers) exposed to the chemicals only after weaning for two years; (2) studying the effects on reproduction, behavioral, endocrine, and immunological functions at specific time periods during the bioassay in additional groups of animals (incorporated in the bioassay design); and (3) the bioassay will be carried out at 3 dose levels plus controls to determine if there is a dose-response relationship of the chemical for carcinogenic and other toxicologic endpoints.

METHODS EMPLOYED: Under this contract three chemicals (diphenylhydantoin, ethylenethiourea, polybrominated biphenyls) are being studied in both male and female B6C3F₁ mice and F344 rats. The project is divided into two phases, prechronic and chronic.

Prechronic Phase of Study: The acute toxicity and MTD (Maximum Tolerated Dose) determination is carried out by following the "Guidelines for Carcinogenic Bioassay in Small Rodents" prepared by the Division of Cancer Cause and Prevention, NCI, NIH.

The Maximum Neonatal Dose (MND) will be determined as follows: mature females (7-9 weeks) will be divided into five groups: (1) control; (2) MTD (as determined in 90 day studies); (3) 1/3 MTD; (4) 1/10 MTD; and (5) 1/30 MTD. Twelve females per group will be dosed with the chemical (in diet) before breeding. These groups will be bred to untreated, proven fertile males after four weeks or to time of steady state for the chemical and continued on the chemical through weaning of the F₁ generation. The size of litters will be limited to 8 in rats

and 6 in mice by random killing of excessive animals on day 4. The MND will be the highest dose at weaning which does not depress the body weight of the offspring more than 10% as compared to the controls; and does not produce mortality, clinical signs of toxicity, pathologic lesions or malformations that would be predicted to shorten the animals' natural lifespan. Extra animals will be added in MND determination experiments to analyze the chemical and/or its metabolite(s) concentrations in the tissues and body fluids by using standard analytical GLC or mass spectrometry methods.

Chronic Phase of Study: In addition to the development of tumors as an end point, the appropriate toxicity tests, general histopathology, behavioral and immunology function tests will also be performed on parallel sets of animals placed on same dose regimen at specific time intervals during the test period. The chronic phase will begin with 4 groups of sexually matured (7-9 weeks) females of both species; groups of 90 animals will receive MND of the chemical; two groups of 30 animals in each will receive 1/3 MND and 1/10 MND respectively; the fourth group of 90 animals will not receive any treatment. The dosing will begin 4 weeks later, or to the time of steady state for the chemical, before breeding of all groups. Three days before anticipated delivery, the animals will be transferred to a suitable cage to litter. The F₀ females will continue to receive the test chemical while nursing their litters.

- A. Carcinogenicity Testing - At weaning of above four groups, not more than 2 males and 2 females, shall be selected randomly from each litter to obtain the total required for the carcinogen bioassay. Eight groups (16 for both sexes) consisting of 50 offspring in each (derived from F₀ mothers) will be treated with test chemical for 2 years as outlined below:

F ₀ Treatment Group	F ₁ Offspring Randomized Grouping	F ₁ Treatment
	_____	MTD
MND	_____	1/3 MTD
	_____	No treatment
1/3 MND	_____	1/3 MTD
1/10 MND	_____	1/10 MTD
	_____	MTD
Untreated	_____	1/3 MTD
	_____	Control

For evaluation of carcinogenic potential the contractor will follow specific toxicopathologic procedures suggested by NIEHS.

B. General Toxicology Tests - A number of tests will be performed on separate animals incorporated in the carcinogen bioassay design. These animals will be exposed to the test chemical at the same dose regimen as that of carcinogen bioassay groups. Various toxicologic endpoints to be tested are described below:

1. Toxicopathologic Evaluation - A parallel set of 8 groups of each sex shall be set up. These groups will consist of 10 male and 10 female animals at each test level. Each group shall consist of one F₁ male and one F₁ female randomly selected from each 10 litters. These groups will be placed on the appropriate treatment at weaning and sacrificed at 9 months of age for toxicopathologic evaluations which include gross pathology, histopathology, clinical chemistry and tissue levels of the test chemical.
2. Reproductive Function Tests - The animals of the toxicopathologic group, prior to their sacrifice, will be subjected to reproductive function tests.
3. Immune-Function Tests - One additional set of eight groups will consist of 12 males at each test level. Each group will consist of one F₁ male randomly selected from each of 12 litters at weaning and placed on the appropriate treatment. The animals will be sacrificed at 9 months for immune-function tests such as: T-cell function, in vitro by assessing the response of splenic or peripheral blood lymphocytes to mitogen concanavalin A and/or phytohemagglutinin; in vitro B-cell function by assessing its in vitro response of lymphocytes to poke weak mitogen or E. Coli lipopolysaccharide; antibody response to T-dependent antigen by plaque assay; delayed hypersensitivity reaction; and quantitation of immunoglobulin.
4. Behavioral Tests - A battery of behavioral tests will be applied to 10 male rats per treatment groups from 10 separate litters and 20 male controls from separate litters. The behavioral tests will be performed in the same animals at 4 weeks, 9 months, and 2 years of carcinogen bioassay groups. The tests will include spontaneous motor activity; presence or absence of autonomic signs and for the appearance of normal or deferred motor and pain reflexes; visual placement responses; forelimb grip strength; hind limb extensor reflexes; startle responsiveness and habituation to a time-locked acoustic signal; and one-way avoidance response.

MAJOR FINDINGS AND PROPOSED COURSE: There were originally four chemicals (phenytoin [diphenylhydantoin], ethylenethiourea, Firemaster FF-1 [polybrominated biphenyls mixture][®] and Kepone[®]), that were selected for study under this contract. However, due to the budgetary constraints, Kepone[®] was withdrawn. The following is the status of study on the individual chemicals:

Phenytoin: The in-life chronic phase is complete. The histopathologic evaluations at the laboratories are underway.

Ethylenethiourea: The in-life chronic phase of this study is complete. The histopathologic evaluations at the laboratories are underway.

Firemaster FF-1: The chronic phase on this chemical is underway.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The discovery of cancer in the daughters of women exposed to diethylstilbesterol, toxicity in infants exposed to hexachlorophene, or morphologic and functional impairments in children whose mothers were exposed to environmental mercury emphasizes the significance of prenatal exposure to chemicals and resulting delayed toxicologic or carcinogenic effects in offspring. The objective of this program is to test the concept that prenatal plus postnatal exposure of chemicals for carcinogen bioassay is perhaps a more sensitive method for assessment of toxicity and carcinogenicity of selected chemicals as compared to widely used approach of lifetime exposure of young adult animals. This research project is directly relevant to the mission of the National Toxicology Program implemented by NIEHS. The data from this research will aid in better risk assessment of the selected chemicals for human exposure for a wide spectrum of the population as well as strengthen the need for different approaches necessary for recognition of reversible or irreversible toxic properties of chemicals in general or for a class of chemicals.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20816
(NIH-N01-ES-95650)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals for Sodium Azide, Tris(2-chloroethyl)phosphate and d,l-Amphetamine Sulfate

CONTRACTOR'S PROJECT DIRECTOR: Lois T. Mulligan, Ph.D.

PROJECT OFFICER (NIEHS): Kamal M. Abdo, Ph.D., Chemist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 29, 1980

CURRENT ANNUAL LEVEL: \$31,452

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Sodium Azide - The laboratory materials and methods reports, and draft pathology narrative were received by NTP for quality assurance and pathology working group evaluation.

Tris(2-chloroethyl)phosphate - The laboratory materials and methods reports, and draft pathology narrative were received by NTP for quality assurance group evaluation.

d,l-Amphetamine Sulfate - The in-life phase of the chronic studies in rats and mice is complete. The pathology phase is expected to be completed by November, 1985.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-95651-01)

TITLE: Toxicity and Carcinogenicity Studies in Laboratory Animals on Furan, Furfural, Gamma-Butyrolactone, Benzaldehyde, Furfuryl Alcohol, and Hexachlorocyclopentadiene

CONTRACTOR'S PROJECT DIRECTOR: J.David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: June 30, 1980

CURRENT ANNUAL LEVEL: \$300,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Furan - Chronic histopathology in progress.

Furfural - Chronic histopathology in progress.

Gamma-Butyrolactone - Chronic histopathology in progress.

Benzaldehyde - Chronic histopathology in progress.

Furfuryl Alcohol - Prechronic tests complete; chronic terminated.

Hexachlorocyclopentadiene - Prechronic tests complete; chronic terminated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

PUBLICATIONS

Abdo, K.M., Montgomery, C.A., Kluwe, W.M., Farnell, D.R., and Prejean, J.D.: Toxicity of hexachlorocyclopentadiene: subchronic (13-week) administration by gavage to F344 rats and B6C3F₁ mice. J. Appl. Toxicol. 4: 75, 1984.

Farnell, D.R., Montgomery, C.A., Giles, H.D., Irwin, R.D. and Prejean, J.D.: Animal model of human disease cirrhosis: Induction of fibrosis and cirrhosis by furan in rats and mice. J. Vet. Pathology (in press), 1985.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-95653-01)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals:

Task Order 01 (Ochratoxin A, Parachloroaniline, Dimethoxane);

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE TASK ORDER INITIATED: August 31, 1980

CURRENT ANNUAL FUNDING: \$56,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

N01-ES-95653-01: All laboratory work on these chemicals should be completed this year. Contract expiration date is July 31, 1985.

Ochratoxin A - This study used rats only. The prechronic studies indicated an effect on the liver. The chronic sacrifice was September, 1984. There were 3 dose groups plus controls with interim sacrifices at 9 and 15 months. Urinalysis, hematology, clinical chemistry and renal function tests were performed. Pathology evaluation is underway.

Parachloroaniline - Animals were sacrificed in February, 1984. Special hematology and methemoglobin studies were performed. Chronic final report was submitted to NTP in September, 1984.

Dimethoxane - Chronic study included an interim sacrifice at 15 months including hematology and selected clinical chemistry on these animals. The terminal sacrifice was August, 1984. Pathology is in progress. Chronic final laboratory report has been submitted to the NTP.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-95653-02)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals:

Task Order 02 (Vinylcyclohexene Diepoxide, Ethylenediamine,
N-Methylolacrylamide);

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE TASK ORDER INITIATED: September 30, 1980

CURRENT ANNUAL FUNDING: \$11,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

NO1-ES-95653-02: All laboratory work on these studies should be completed this year. Contract expiration date is June 30, 1985.

Ethylenediamine - Only the prechronic was performed. This study was completed in February, 1982.

N-Methylolacrylamide - Chronic study had no interim sacrifices and no special studies included. Animals were sacrificed in April, 1984. Pathology is in progress.

Vinylcyclohexene diepoxide - Chronic study includes a 15 month interim sacrifice with hematology performed on these animals. Sacrifice occurred in October, 1984. Pathology is in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

BATTELLE MEMORIAL INSTITUTE
COLUMBUS LABORATORIES
Columbus, Ohio 43201
(NIH-N01-ES-95653-03)

TITLE: Carcinogenicity and Toxicity Studies in Laboratory Animals:

Task Order 03 (o-Benzyl-p-Chlorophenol, Tricresyl Phosphate, prechronic studies only).

CONTRACTOR'S PROJECT DIRECTOR: Arthur C. Peters, D.V.M.

PROJECT OFFICER (NIEHS): William C. Eastin, Jr., Ph.D., Physiologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE TASK ORDER INITIATED: September 30, 1981

CURRENT ANNUAL FUNDING: \$56,000 for the period beginning 9/30/81

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

N01-ES-95653-03: All work on these studies is complete. Contract expiration date is May 30, 1985.

o-Benzyl-p-chlorophenol - This prechronic study was completed in October, 1983. A special immunotoxicology study was initiated June 17, 1984 and completed on July 13, 1984 and the report was submitted to NTP in September, 1984.

Tricresyl Phosphate - The prechronic study was completed in 1983.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

SOUTHERN RESEARCH INSTITUTE
Birmingham, Alabama 35255
(NIH-N01-ES-95656-02)

TITLE: Toxicity and Carcinogenicity Studies in Laboratory Animals for Polysorbate 80, Ethylene Glycol, C.I. Pigment Red 3, and C.I. Pigment Red 23

CONTRACTOR'S PROJECT DIRECTOR: J. David Prejean, Ph.D.

PROJECT OFFICER (NIEHS): Jeffrey J. Collins, Ph.D., Toxicologist
Carcinogenesis and Toxicology Evaluation Branch/TRTP

DATE CONTRACT INITIATED: September 30, 1980

CURRENT ANNUAL LEVEL: \$300,000

PROJECT DESCRIPTION

OBJECTIVES: Within the past 25 years the number of chemicals introduced into the environment has increased dramatically. Surprisingly, there is relatively little known about the toxicologic and/or carcinogenic potential of many of them, even though their use may be widespread and the potential for human exposure is relatively high. The National Toxicology Program is attempting to fill this gap in knowledge through the sponsorship of well-defined bioassay evaluations on a number of these materials. As part of this program, the studies conducted under these contracts were designed to establish basic toxicity data on specific chemicals, using Fischer 344 rats and B6C3F₁ mice. Then, based on the data obtained, additional testing in the form of long-term toxicologic/carcinogenic evaluations are pursued in order to provide sufficient data to establish the relative safety or hazard to humans.

METHODS EMPLOYED: Prechronic (repeated-dose and subchronic) investigations conducted under these contracts include the extensive use of clinical chemistry evaluations, hematology profiles, sperm morphology and vaginal cytology evaluations, and histopathology. Design of the chronic studies varies depending upon the prechronic data, however, the basic format used consists of the administration of 2 or 3 dose levels to groups of 50 animals/sex/species for 103 weeks followed by a one-week observation period. Matched control groups are included in each study. In some cases, additional dose levels, additional animals for interim evaluation, and certain special studies (hematology and clinical chemistry profiles, etc.) are also employed. Histopathologic evaluation is performed on all animals in each study. All studies are being conducted in compliance with the FDA Good Laboratory Practice Regulations. In-life and histopathologic observations are being collected by computer using the NTP Toxicology Data Management System. Other data, including gross observations at necropsy, are collected using manual methods.

MAJOR FINDINGS AND PROPOSED COURSE:

Polysorbate 80 - Chronic histopathology in progress.

Ethylene Glycol - Chronic histopathology (mouse) in progress; chronic rat study terminated.

C.I. Pigment Red 3 - Chronic histopathology in progress.

C.I. Pigment Red 23 - Chronic histopathology in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Results of these studies will provide information about the toxicity of chemicals in experimental animals, which can be used to predict possible hazard to humans, and determine dose levels for a chronic study, if needed.

PUBLICATIONS

Melnick, R.L.: Toxicities of ethylene glycol and ethylene glycol monoethyl ether in Fischer 344/N rats and B6C3F₁ mice. Environ. Hlth. Persp., 59: 147, 1984.

CELLULAR AND GENETIC TOXICOLOGY BRANCH

CELLULAR AND GENETIC TOXICOLOGY BRANCH

Summary Statement

The Cellular and Genetic Toxicology Branch functions to improve our understanding of the mechanism of cellular and genetic toxicity and to develop and use test systems that can identify and characterize chemicals that have potential genetic toxicity. The development and evaluation of test systems currently includes methods to measure mutagenicity in microbial and mammalian cells and *Drosophila*, chromosomal changes (aberrations, SCEs, aneuploidy) DNA damage or neoplastic changes in eukaryotic cells. Test results will be used to define potential genetic toxicity, to select chemicals, to set priorities for further testing and to aid in the design and interpretation of long-term animal carcinogenicity and toxicity studies.

An important goal of the Branch effort, therefore, is to establish a scheme of short-term testing that can be used to predict carcinogenicity and germ cell mutagenicity of chemicals, and, thereby, reduce the need for in vivo assays, or to assist in setting testing priorities for long-term animal assays. However, for short-term tests to be predictive, several criteria must be fulfilled. These include a knowledge of the reproducibility of individual test results, the accuracy with which test results from single or multiple tests predict a toxic effect in vivo, and the relationship of the endpoint measured to carcinogenicity, mutagenicity, or other manifestations of in vivo toxicity. The application of a group of complementary tests that meet these criteria may ultimately result in an effective system for testing chemicals. An important part of the Branch program is to produce sufficient short-term test data, particularly across chemical classes, to establish the accuracy with which one can predict carcinogenic effects in animals. Even with the appropriate use of available test systems, some potential carcinogens (or cocarcinogens or tumor promoters) may not be identified, particularly those that do not induce damage leading to observable gene mutations or chromosomal changes. Therefore, it is important that we continue to develop new methods that are capable of detecting carcinogens that are not identified by the assays currently in use and to identify chemicals that "promote" tumor development. In order to accomplish these goals, it is important that the program remain involved in, and responsive to, basic research developments.

A substantial portion of Branch resources are committed to studies of chemically induced mutations. These mutation studies can be divided into two categories: somatic cell and germ cell. The major difference between the two is that mutations arising in germ cells can be transmitted to subsequent generations, while somatic cell mutations are expressed only in the affected individual. Somatic cell mutagenicity test systems are considered relevant for germ cell mutation because they measure mutagenicity in mitotically dividing cells. In addition, the information gained from tests using somatic cell assay systems has implications for heritable mutation risk because a chemical that is mutagenic in somatic cells may have the potential to be mutagenic in germ cells. By the same reasoning, germ cell mutagens are likely to be somatic cell mutagens.

The portion of the program concerned with validation of assays and the testing of chemicals is performed primarily through extramural contracts and interagency agreements. Basic research, the development or modification of tests, and the management and analysis of data, are generally performed within NIEHS.

The key extramural contract activities include: mutagenesis testing in *Salmonella*, *Drosophila*, and mammalian cells (gene mutations, chromosome aberrations (CA's), and sister-chromatid exchanges (SCE's)); the development and evaluation of an in vivo assay to detect cytogenetic damage; the development of assays for induction of aneuploidy in yeast and *Drosophila*; an in vitro assay in mammalian cells for induced DNA damage; and an evaluation of three mammalian cell transformation systems, including Syrian hamster embryo (SHE) cells, SHE cells infected with Simian adenovirus (SA7), and retrovirus-infected rat cells using coded compounds in at least two laboratories. (The latter two systems measure chemical enhancement of viral transformation or viral-mediated transformation.) Additional studies include the development of assays to detect chemically induced gene transposition in *Drosophila* and mammalian cells and to develop or obtain a human cell system that can be used to test the genetic toxicity of chemicals.

A coordinated testing effort is being conducted to acquire test results from several short-term tests on a group of carcinogens and, in particular, noncarcinogens in order to determine better the ability of individual and multiple short-term tests to predict carcinogenicity in rodents.

Other projects include an effort to develop a standardized protocol by which the frequencies of CA's and SCE's can be accurately and reproducibly measured in human lymphocytes with particular emphasis on understanding the sources of variation that may affect the measurement of these endpoints. The measurement of chemically induced germ cell mutations in mice is done by both the morphological and biochemical specific-locus assays. Other developmental projects include analysis of mutagens produced in cooked foods and an attempt to develop an assay for chemically induced transpositions of specific DNA sequences in *Drosophila*.

Intramural research efforts involve both prokaryotes and eukaryotes. Studies using *Salmonella typhimurium* include attempts to increase our understanding of the *Salmonella* mutagenicity test system, to improve the sensitivity and efficiency of protocols currently in use, and to use the *Salmonella* test as a tool to study in vitro and in vivo metabolism of mutagenic chemicals.

Two aspects of meiotic development are being studied using the yeast *Saccharomyces cerevisiae* as a model genetic and biochemical system. The first concerns the role of DNA repair mechanisms in meiosis; several mitotically defined repair genes are essential in normal meiotic/germinal processes. The second involves identifying those gene functions that are specific to meiosis. Both repair- and the meiotic-specific genes are being isolated, and their role during meiosis, particularly recombination, is being examined. Yeast systems are also being developed to examine chromosomal and gene mutagenesis and the role of DNA repair systems using defined centromere and expressed gene sequences. The susceptibility of centromere region DNA to damage will be related to the unique protein DNA

structure of centromeres. The expressed gene, a cloned tRNA suppressor that can be readily sequenced, will be used to examine the molecular events associated with mutagenesis.

The genetic control of DNA repair and mutagenesis is also being studied in Drosophila melanogaster. In particular, emphasis is placed on a detailed structural analysis of the mei-41 gene, which is involved in post-replication DNA repair, and the mu-2 gene, which controls mutation rates near chromosome ends. The mu-2 gene does not control DNA repair directly, but appears to code for a chromosomal protein that interacts with DNA repair enzymes.

Oncogenesis by transposable elements is being studied in spontaneous and induced hematopoietic neoplasms of the RFM/Un mouse. This model system is based on the somatic reintegration of endogenous provirus in certain neoplastic phenotypes. In order to test the "promoter/enhancer insertion" mechanisms of oncogenesis somatically-acquired provirus integration sites have been cloned and analyzed. Although a survey of several cloned oncogenes has not shown any homology to these integration sites, efforts will be made to identify these sequences and determine if any specific sites are involved. The long-range goal of this project is to identify the genetic targets, possibly oncogenes, that are involved in the development of specific neoplastic phenotypes.

The problem of organ and species specificity of chemical carcinogens is being studied by measuring the production of mutagenic metabolites from primary epithelial cells (activator cells) of liver, lung, and urinary bladder of different species. The activator cells are cocultivated with target cells (V79 hamster cells or Salmonella) to measure toxicity, SCE's, and mutation. Future studies will involve activation of aromatic amines by measuring metabolite production and DNA-adduct formation and repair. Also, the role of prostaglandin synthetase, an enzyme known to contribute to carcinogen activation, will be studied. Long-range plans include development of systems to measure chemically-induced multiple genetic endpoints in human cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21012-04 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Organ and Species Differences in Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Langenbach Microbiologist CGTB, NIEHS

Others: K. Rudo Biologist CGTB, NIEHS

COOPERATING UNITS (if any)

Genetic Toxicology Division, U.S. EPA (James Allen)

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

Carcinogen Metabolism

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.4

PROFESSIONAL

1.4

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An in vitro approach for studying organ and species differences in the activation of chemical carcinogens is being utilized. Intact cells are used for metabolic activation because they simulate in vivo metabolism. To assess biological activity of metabolites, the genetic endpoints, toxicity, mutation and/or SCE induction in V79 cells and reversion of S. typhimurium, are used. In addition, HPLC analysis of metabolites formed by primary cells from different organs and species have been conducted. Work during the past year has investigated differences among rat, dog, and bovine liver and bladder cell activation/metabolism of aromatic amines. Species differences in the relative ability of these organs to activate aromatic amines have been observed. Results from another study indicated differences between rat and hamster hepatocyte activation of nitrosamines and aromatic amines and the relative sensitivities of V79 cell mutation and SCE induction and Salmonella reversion to the activated intermediates. Additionally, structure-activity analyses of short chain aliphatic nitrosamines in the hamster hepatocyte-mediated V79 cell mutagenesis system have been conducted and the possible correlation of mutagenicity with carcinogenicity investigated.

PROJECT DESCRIPTION

METHODS EMPLOYED: The target organisms, V79 cells or *S. typhimurium* are co-cultivated with freshly prepared intact hepatocytes or bladder cells and the multiple genetic endpoints (toxicity, mutation and SCE induction and histidine reversion) measured. In addition, metabolism of chemicals by the primary cells is analyzed by HPLC.

MAJOR FINDINGS AND PROPOSED COURSE: An understanding of the causes for organ and species specificity of chemical carcinogens is fundamental to understanding the mechanism(s) of carcinogenesis and essential for valid extrapolation of rodent carcinogenicity data to estimate human hazard. An *in vitro* approach for studying activation of chemical carcinogens has been previously developed by the principal investigator, and this work is a continuation of these studies.

The major findings are: (1) The relative contribution of liver and bladder from different species (rat, dog and bovine) in the activation of bladder carcinogens has been determined. (2) Differences between hamster and rat hepatocytes in the activation of carcinogenic chemicals from various chemical classes to genotoxic intermediates have been made and sensitivity of various genetic endpoints (i.e., V79 toxicity, mutation, SCE induction and reversion of *S. typhimurium*) have been compared. (3) Structure-activity relationships among short chain aliphatic nitrosamines in the hamster hepatocyte-mediated V79 cell mutagenesis have suggested mechanisms of action of these chemicals and demonstrated the utility of the hepatocyte-mediated system in predicting carcinogens.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The studies provide a methodology for evaluating a major component (metabolic activation) known to determine organ and species specificity. Such data aid in elucidating the mechanisms of specificity in cancer initiation and hopefully, with the use of human cells and the multiple genetic endpoints described, will facilitate extrapolation of bioassay data for predicting human hazard.

PUBLICATIONS

1. Sharief, Y., Campbell, J., Leavitt, S., Langenbach, R., and Allen, J. Rodent species and strain specificities for sister-chromatid exchange induction and gene mutagenesis effects from ethyl carbamate, ethyl N-hydroxycarbamate, and vinyl carbamate. Mut. Res. 126: 159-167, 1984.
2. Tompa, A. and Langenbach, R. Epithelial cell culture of liver, lung, and pancreas for detection of cell-specificity in metabolic activation to mutagens of chemical carcinogens. In Rohlich, P. and Bacsy, E. (Eds.) Tissue Culture and RES. Budapest, Hungarian Academy of Science, 1984, 355-362 pp.
3. Langenbach, R. In vitro systems to study organ and species differences in the metabolic activation of chemical carcinogens. In Huberman, E. (Ed.) The Role of Chemicals and Radiation in the Etiology of Cancer. In press.

4. Nesnow, S., Langenbach, R., and Mass, M. J. Pattern recognition analysis of a set of mutagenic aliphatic N-nitrosamines. Environ. Health Pers. In press.
5. Hatch, G. G., Conklin, P. M., Christensen, C. C., Langenbach, R., and Nesnow, S. Mutation and enhanced viral transformation of cultured hamster cells by exposure to gaseous ethylene oxide. Environ. Mutag. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01-ES 21013-04 CGTB																									
PERIOD COVERED October 1, 1984 to September 30, 1985																											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Analysis of Gene Toxic/Carcinogenic Events in Mammalian Cells																											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">L. R. Boone</td> <td style="width: 30%;">Senior Staff Fellow</td> <td style="width: 10%;">CGTB</td> <td style="width: 10%;">NIEHS</td> </tr> <tr> <td colspan="5" style="padding-top: 10px;">OTHERS:</td> </tr> <tr> <td></td> <td>R. W. Tennant</td> <td>Supervisory Microbiologist</td> <td>CGTB</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>P. L. Glover</td> <td>Bio. Lab. Tech.</td> <td>CGTB</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>C. L. Innes</td> <td>Bio. Lab. Tech.</td> <td>CGTB</td> <td>NIEHS</td> </tr> </table>			PI:	L. R. Boone	Senior Staff Fellow	CGTB	NIEHS	OTHERS:						R. W. Tennant	Supervisory Microbiologist	CGTB	NIEHS		P. L. Glover	Bio. Lab. Tech.	CGTB	NIEHS		C. L. Innes	Bio. Lab. Tech.	CGTB	NIEHS
PI:	L. R. Boone	Senior Staff Fellow	CGTB	NIEHS																							
OTHERS:																											
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	P. L. Glover	Bio. Lab. Tech.	CGTB	NIEHS																							
	C. L. Innes	Bio. Lab. Tech.	CGTB	NIEHS																							
COOPERATING UNITS (if any) Wen K. Yang Biology Division, ORNL																											
LAB/BRANCH Cellular and Genetic Toxicology Branch																											
SECTION																											
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709																											
TOTAL MAN-YEARS: 2.9	PROFESSIONAL: 1.2	OTHER: 1.7																									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																											
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) We have investigated the transposition of endogenous proviral genes in hemato-poietic neoplasms of RFM/Un mice. Our results indicate that somatically acquired provirus is integrated at novel sites in lymphosarcomas of 5-azacytidine treated mice. Analysis of these neoplasms and spontaneous reticulum cell sarcomas with <u>myc</u> , <u>myb</u> and <u>mos</u> oncogene probes has not revealed any genomic rearrangements of these genes. We have molecularly cloned three proviruses from a single RFM/Un spleen DNA sample in order to identify genes at the integration site that may be involved in the neoplastic phenotype. Analysis reveals that one clone is a complete infectious provirus, another is apparently intact but lacks infectivity, and a third has a deletion in the env gene. Restriction enzyme maps have been determined for the viral and cellular flanking regions of these clones. In collaboration with Dr. Miles Cloyd, Duke University, novel retroviruses have been isolated from RFM/Un mice. These apparent recombinant genome viruses may be important components of the etiology of RFM/Un lymphomagenesis. In collaboration with Dr. Wen Yang, Oak Ridge National Laboratory, we have analyzed the structure of endogenous noncotropic proviral genomes molecularly cloned from this strain. The LTR, prime binding site and env region of the endogenous noncotropic proviruses are distinct from the inducible ecotropic provirus. The contribution of these endogenous elements to lymphomagenesis in the RFM/Un mouse is being further studied by molecular and biological techniques.																											

PROJECT DESCRIPTION

METHODS EMPLOYED: Recombinant DNA cloning and restriction enzyme mapping has been employed to isolate and characterize somatically acquired proviruses, and biological activity has been determined by DNA transfection into NIH 3T3 cells. Flanking regions of the somatically acquired proviruses have been subcloned to provide probes for the identification of the genomic location of these proviruses. Oncogene probes have been employed to determine if rearrangements of these genes are involved in the RFM/Un lymphomas.

MAJOR FINDINGS AND PROPOSED COURSE: We have continued our analysis of proviruses in spontaneous reticulum cell sarcomas and radiation induced myeloid leukemia in the RFM/Un mouse. Previous results revealed a strong correlation between somatically acquired ecotropic provirus and reticulum cell sarcoma. Many of the proviruses are defective, rearranged, or possibly recombinants as evidenced by altered restriction fragment sizes relative to the presumed progenitor endogenous ecotropic provirus. Analysis with a molecular probe specific for the env gene of leukemogenic MCF virus does not reveal amplification or rearrangement of this class of viral sequences. Likewise, the oncogene probes *myc*, *myb* and *mos* have not detected any genomic rearrangements that would implicate these genes. Future analysis will involve additional oncogene probes and integration site specific probes.

Recently, 5-azacytidine treated RFM/Un mice were examined in a similar manner and somatically acquired ecotropic provirus was detected in lymphoid neoplasms. We have molecularly cloned three proviruses from a single spleen DNA sample. One clone, a 9.5 Kb EcoRI fragment, is infectious following DNA transfection of NIH-3T3 cells. We will analyze the host range properties of this isolate as it may lack sensitivity to the RFM restriction mechanism. Another clone (10 Kb EcoRI fragment) has a deletion in the env gene and is not infectious. Flanking region probes will be prepared as they have been for the proviruses from myeloid leukemia, and used to identify common integration sites in lymphoma sarcomas.

In collaboration with Dr. Miles Cloyd, Duke University, a novel virus class has been isolated from the spleen of 5-azacytidine treated RFM/Un mice. Biological and molecular analysis indicates that the virus has a distinct env gene and is likely to be a recombinant between the inducible RFM endogenous ecotropic virus and some other endogenous provirus. This virus may have an important role in the RFM/Un lymphomagenesis and we will continue our collaboration with Dr. Cloyd during the coming year in order to further characterize this isolate.

In collaboration with Dr. Wen Yang, Oak Ridge National Laboratory, we have continued our analysis of the structure of endogenous proviruses in the RFM/Un mouse. The prime binding site of five proviruses has been found to be tRNA *glu* rather than tRNA *pro* like the endogenous ecotropic proviruses. We will continue to collaborate with Dr. Yang in the coming year with emphasis on analysis of the solitary LTRs of RFM/Un.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE: The significance of our findings is that it may identify a common genetic target, possibly an oncogene, that is specific for a neoplastic phenotype. Identification of specific sequences involved in carcinogenesis is of considerable importance in contemporary biomedical research. This project is aimed at providing basic information concerning the mechanism of gene rearrangements and/or structural alteration that contribute to carcinogenesis. The ability to detect such events in a sensitive and precise manner, and the ability to identify chemical and physical agents with the potential to cause such effects are fundamental goals of the CGTB/NIEHS.

PUBLICATIONS

Nikbakht, K.N., OU, C.-Y., Boone, L. R., Glover, P. L., and Yang, W. K. Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding site for glutamine tRNA. *J. Virol.* 54: 889-893 (1985).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21016-04 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Enzymes Involved in DNA Repair and Meiosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Michael Resnick	Research Geneticist	CGTB	NIEHS
Others:	Terry Chow	Visiting Associate	CGTB	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

1.3

PROFESSIONAL

1.0

OTHER

0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The RAD52 gene in Saccharomyces cerevisiae controls the repair of ionizing radiation-induced DNA double-strand breaks, radiation-induced spontaneous mitotic recombination, and recombination during meiosis. Utilizing an antibody raised against a Neurospora crassa deoxyribonuclease, we observed that in logarithmically growing wild type yeast strains, approximately 80% of Mg⁺⁺ dependent pH 8 single-strand deoxyribonuclease is antibody precipitable. As cells enter stationary phase the antibody-precipitable nuclease decreases to an undetectable level as does cross-reacting material. The antibody precipitable nuclease activity increases five to ten times during meiosis during the period of DNA synthesis and recombination, and decreases at the end of meiosis. No activity is observed in rad50 or rad52 mutants; these results are correlated with the lack of recombination in these mutants. The nuclease that is detected with the antibody has been purified nearly 1000-fold and has a molecular weight of 70,000. It is a single-strand endo-exonuclease which also exhibits double-strand nuclease activity.

Our observations implicate this exo-endonuclease in repair processes, spontaneous and damage-induced mitotic recombination, and normal meiotic recombination. Using a λ gt11 vector expression library that contains genomic yeast DNA and the antibody as the probe, we have identified a segment of DNA that codes for cross reacting material. This will enable us to identify the gene and determine directly the role(s) of the nuclease in recombination and repair.

PROJECT DESCRIPTION

METHOD EMPLOYED: Various repair-deficient strains of *Saccharomyces cerevisiae* are grown using standard techniques. To obtain crude extracts, cells are broken open with a French pressure cell, centrifuged, and the supernatant examined for nuclease activity. The measurement of a RAD52 controlled alkaline deoxyribonuclease involved the use of a rabbit antiserum which was raised against a *Neurospora crassa* single-strand DNA-binding endo-exonuclease. Nuclease activity toward single-strand ³H-labelled DNA was assayed. Cross-reactive material was examined with a dot hybridization or Western blot method, and the purification of the alkaline deoxyribonuclease involved standard column chromatography techniques. Standard biochemical procedures were used to characterize the deoxyribonucleases. The cloning of the nuclease gene involves standard bacterial and yeast vectors and cloning procedures.

MAJOR FINDINGS AND PROPOSED COURSE: DNA repair processes are required to protect against external damaging agents and many of the repair mechanisms are involved in mutagenesis and recombination. In *Saccharomyces cerevisiae*, the RAD52 gene has been genetically identified as controlling DNA repair of ionizing radiation damage, EMS mutagenesis, and spontaneous and induced recombination. It and RAD50, which is also involved in DNA double-strand break repair, are essential in meiotic recombination. We recently identified a nuclease activity in crude extracts of RAD⁺ cells that can be removed by an antiserum raised against a *Neurospora crassa* single-strand endo-exonuclease. Since rad52 strains lack any antibody precipitable nuclease activity, and at most 10% of the cross-reacting material, we concluded that the RAD52 gene is involved with the appearance of this activity. Considering the central role that RAD52 plays in repair and recombination, this nuclease could have an important function in these processes. We are pursuing the nature and function of this enzyme using several genetic, molecular, and enzymological approaches.

Using sensitivity to antibody precipitation we have been able to follow the purification of this single-strand alkaline (pH 8) deoxyribonuclease to obtain nearly a 1000-fold purification. No antibody precipitable nuclease activity was detected when extracts of rad52 cells were subjected to similar purification procedures. The resulting protein appears to have a molecular weight of 72,000 and requires Mg^{++} for activity. Recent unpublished reports of Ogawa have described an open reading frame for the RAD52 gene of 1500 base pairs which would correspond to a molecular weight of 63,000. Furthermore, null rad52 mutants did not alter the position of the much reduced 72 Kd protein on Western blots. Thus the antibody precipitable nuclease does not correspond to the product of the RAD52 gene. The enzyme is a single-strand endo-exonuclease based on its ability to degrade single-strand DNA and nick both supercoiled DNA and gapped M13 molecules. It also degrades double-strand DNA and RNA. The action on ssDNA is non-processive but is processive on dsDNA. The ssDNase and dsDNase activities appear to be associated with the same protein based on a common heat inactivation pattern at 40°C. The enzyme therefore appears to have many of the properties expected of a nuclease involved in repair and recombinational processes.

Both precipitable enzyme activity and cross-reacting material are detected in logarithmically growing haploid and diploid cells. No significant activity is

observed in stationary cells; this is consistent with observations of little or no cross-reacting material in crude extracts or in SDS polyacrylamide gels. Cells growing logarithmically in presporulation medium containing acetate as a carbon source also exhibit activity. However, late log cells which are used in meiosis experiments have no antibody precipitable nuclease activity. The antibody precipitable nuclease increases shortly after transfer of the log cells to meiotic medium. Its activity reaches a peak at about the time of recombination. No precipitable activity is observed in rad52 mutants throughout meiotic development. These results along with observations of lack of recombination and lethality in rad52 suggest that the nuclease is important in the recombinational events of meiosis. This is the first case of an enzyme being specifically identified with meiotic recombinational processes. The absence of this nuclease activity in mitotically growing rad52 mutants also implicates it in RAD52 mediated repair processes.

To identify the gene that codes for the nuclease, we screened an expression library using the antibody as a probe. Utilizing a Agtl1 expression vector, a 4 Kb segment of yeast DNA was identified which contained at least a portion of DNA that codes for the cross-reacting materials. We are currently using this 4 Kb segment of DNA as a probe to obtain the complete gene from a YEP213 yeast genomic library.

Ongoing and future research plans are devoted to the further characterization of the enzyme, determining its presence under various growth conditions and following exposure to DNA damaging agents, examining it in several repair mutants, and further cloning and sub-cloning of the 4 Kb yeast genomic DNA for the corresponding gene. We are also examining other organisms and mammalian cells for a similar cross-reacting nuclease. To aid in these studies we are raising antibody to the purified yeast nuclease.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In yeast as well as other eukaryotes, DNA repair and recombination systems can have common control mechanisms based on genetic evidence. The RAD52 gene is essential in DNA repair, mutagenesis with certain agents, normal chromosomal disjunction, and mitotic and meiotic recombination. An understanding of the function of the RAD52 gene and associated gene product(s) will enhance our understanding of all these processes. The techniques and antibody probes which have been developed are being used to investigate similar mechanisms in cells of higher organisms.

PUBLICATIONS

Resnick, M.A., Chow, T., Nitiss, J., and Game, J.: Changes in the chromosomal DNA of yeast during meiosis in repair mutants and the possible role of a deoxyribonuclease. Cold Spring Harbor Symposium on Quantitative Biology, 49: 639-649, 1984.

Resnick, M.A., Sugino, A., Nitiss, J., and Chow, T.: DNA polymerases, deoxyribonucleases and recombination during meiosis in yeast. J. Molec. Cell. Biol. 4: 2811-2817, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21032-01 CGTB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Development of Peroxidase Oxidation Systems in Mutation Assays		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 30%;"> PI: W. Caspary Others: D. Daston </div> <div style="width: 30%;"> Biochemist Biologist </div> <div style="width: 30%;"> CGTB, NIEHS CGTB, NIEHS </div> </div>		
COOPERATING UNITS (if any) Laboratory of Molecular Biophysics, NIEHS		
LAB/BRANCH Cellular and Genetic Toxicology Branch		
SECTION Chemical Mutagenesis		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.5	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Mechanisms of metabolism other than those mediated by the mixed fraction oxidases may be important in activating certain chemicals to their ultimate carcinogenic form. Recent evidence suggests that benzidine dyes are cleaved by intestinal bacteria, thereby liberating the parent benzidine and its congeners. There is evidence implicating peroxidases in the metabolism of benzidine dyes.</p> <p>Prostaglandin H synthetase will be used to activate compounds in the mouse lymphoma forward mutation assay. The possible mechanisms responsible for the formation of mutagenic metabolites induced by various peroxidase enzyme systems are being investigated. The use of selective inhibitors of the peroxidase are being used to aid in the elucidation of these mechanisms. Studies include the identification of metabolites induced by this activation system.</p>		

PROJECT DESCRIPTION

MAJOR FINDINGS AND PROPOSED COURSE: Many compounds are activated to their carcinogenic form by the mixed function oxidases. However, it is clear that some compounds are activated by other enzymes that are part of the mixed function oxidase system. Such enzymes are the peroxidases. We expect to determine conditions under which certain model compounds such as aromatic amines are oxidized by prostaglandin H synthetase to forms that induce mutations in the mouse lymphoma cell system. Once such conditions are found, metabolites and DNA adducts will be analyzed to determine the mechanism of action of the mutagenic activity of these compounds via this metabolic route.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: While the mixed function oxidases are responsible for the metabolism of many xenobiotics, it is becoming clear that other metabolic routes that produce short-lived metabolites may be important in inducing toxicity in the host. These metabolites react with macromolecules in the host to produce neoplasia, mutations, and teratogenesis. Prostaglandin H synthetase is found in many mammalian tissues, especially the lung and the kidney. Exploration of mechanisms of action of prostaglandin H synthetase induced metabolites may identify important metabolic routes that are important in these disease states. In addition, the development of such an exogenous metabolic activation system in the presence of a mammalian cell should aid in the development of short-term assays used by CGTB in screening for the potential mutagenic and carcinogenic effects of chemicals.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21035-01 CGTB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structural Analysis of Meiotic Chromosome Behavior in Yeast and the Mouse		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I.: C.N. Giroux Senior Staff Fellow CGTB NIEHS		
COOPERATING UNITS (if any) Michael Dresser, Duke University, Durham, North Carolina Montrose Moses, Duke University, Durham, North Carolina		
LAB/BRANCH Cellular and Genetic Toxicology Branch		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided) <p>The focus of this project is to investigate at the molecular level the structural basis of <u>meiotic chromosome</u> metabolism and segregation in the <u>yeast, Saccharomyces cerevisiae</u>, and to compare it to that of the <u>mouse and related mammalian species</u>. A combined cytogenetic and immunochemical analysis is being used to identify protein components of the <u>synaptonemal complex</u> and to determine their spatial distribution and organization. <u>Methods of isolation and identification by light microscopy</u> are being developed for the yeast synaptonemal complex, as well as for the meiotic spindle apparatus.</p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: Standard methods for growth and sporulation of yeast are used. Examination of yeast material in the light microscope is by phase contrast, dark field, or epi-fluorescence (DAPI or fluorescein label). High resolution examination of yeast and mouse specimens is by transmission electron microscopy procedures following fixation and silver staining. Standard immunological and immunocytological methods are used; antibodies are fluorescently labelled by the double or second antibody method. Proteins are displayed by SDS PAGE followed by Western blotting.

MAJOR FINDINGS AND PROPOSED COURSE: Meiosis is a universal and highly conserved process whereby eukaryotic germ line cells transmit their genetic information to the next generation. The mechanism(s) underlying this differentiation event are unknown. In virtually all meiotic systems, a specific structure, the synaptonemal complex, is transiently produced. This structure has been implicated in proper chromosome metabolism and stability during meiosis; it is suggested that environmental treatments which disrupt or interfere with the synaptonemal complex lead to infertile gametes or aberrant chromosome segregation. In order to investigate the role of the synaptonemal complex in meiotic stability, we have initiated a structural analysis at the molecular level.

There are two initial specific goals of this analysis. First, methods will be developed, based on the surface spreading methods and silver staining procedures of Dresser and Moses, which allow light microscope identification of the yeast synaptonemal complex. Our initial attempts suggest that this aspect can be successful, but will provide limited resolution of structural detail. To improve the level of resolution, we are developing monoclonal antibody probes to the synaptonemal complex. The second initial goal of this project is to develop isolation and immuno-staining procedures which allow direct detailed examination of the synaptonemal complex and other structural elements present in yeast meiotic cells. Pilot experiments using anti-yeast tubulin and DAPI staining of meiotic preparations have allowed a visualization of basic elements of chromosome movement along the meiotic spindle in yeast. Dresser and Moses have isolated monoclonal antibodies which react specifically with the synaptonemal complex in meiotic cells of the mouse and related mammals. We have determined by light microscopy/immunofluorescence that these antibodies react with yeast meiotic cells. Furthermore, initial Western blots of yeast and mouse meiotic cell protein demonstrate that there are shared antigens between the two species.

Based on these preliminary observations, we propose to identify the specific proteins identified in yeast (and mouse) meiotic cells by Western blotting. We further propose to develop and refine our initial cytogenetic characterization of the yeast synaptonemal complex to allow reproducible resolution of structure and immunochemical localization of specific proteins. A comparison between the mouse and yeast synaptonemal complexes will be made.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The structural organization and functional role of specific structures in differentiating meiotic cells have not yet been fully determined. Our preliminary experiments represent the first specific immuno-cytological identification of the spindle apparatus in yeast meiotic cells. We are attempting to extend this methodology to a structural analysis of the synaptonemal complex. Such an analysis will be used to characterize mutants which are defective in meiotic DNA metabolism and to aid in the identification and analysis of meiosis-specific proteins. This fundamental information is necessary for an understanding of the structural elements which are required for proper DNA metabolism and chromosomal stability during meiosis and the differentiation of germ cell lines.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21037-01 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of Salmonella Mutagenicity Testing Results

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	Errol Zeiger	Supervisory Microbiologist	CGTB	NIEHS
Others:	Ken Risko	Mathematical Statistician	BRAP	NIEHS
	Barry Margolin	Mathematical Statistician	BRAP	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

0.5

PROFESSIONAL

0.5

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The database derived from the testing of approximately 1000 chemicals for mutagenicity in Salmonella typhimurium is being analyzed. These chemicals were tested in 4 Salmonella strains without metabolic activation, and with 2 different S-9 preparations, for a total of 12 strain/activation combinations, and some of the chemicals were tested in more than one laboratory. We therefore have the opportunity to analyze numerous parameters. Analysis of the effectiveness of different testing strategies for mutagen detection has shown that TA100, alone, is the most effective strain. A number of 2- and 3-stage testing schemes have been proposed which are more efficient and cost-effective than the use of all tester strain/activation combinations simultaneously. Additional analyses of the database are planned or in progress.

PROJECT DESCRIPTION

METHODS EMPLOYED: The database of Salmonella test data and analyses is maintained on the NIEHS VAX computer. Various statistical and nonstatistical analyses are applied to this database.

MAJOR FINDINGS AND PROPOSED COURSE: Analysis of the effectiveness of different Salmonella testing strategies has confirmed our intuition that TA100 is the most effective strain; it, alone, was able to detect 80% of those chemicals judged to be mutagenic by any strain. The addition of TA98 or TA1535 to TA100 was equally effective; they raised the proportion of mutagens detected to 89% and 88%, respectively. Each strain/activation combination had at least one chemical that was judged mutagenic in that combination alone.

Sequential testing strategies were devised which led to the detection of 100% of the mutagens at a reduced effort. The most effective 2-stage strategy led to an estimated saving of 25% and the most effective 3-stage strategy led to an estimated saving of 30% over the cost of a single-stage protocol. When a sub-population of nitro-containing chemicals was analyzed, similar results were obtained, but, because there was a higher proportion of mutagens in this sub-population, the estimated savings were greater - 64% for the optimal 2-stage strategy and 70% for the optimal 3-stage strategy. Based on our calculations and considerations of laboratory efficiency it is our recommendation that, for screening chemicals for mutagenicity in Salmonella, a sequential scheme be adopted whose initial stage consists of TA100.

The database is being used for additional analyses, which include an examination of the predictivity of one strain for the others; the relationship between the magnitude of the mutagenic response and carcinogenicity; the relationship between the strain/activation mutagenic responses and carcinogenesis; and others.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The NTP, other government programs, and many industrial laboratories use the Salmonella mutagenicity assay to screen large numbers of chemicals for mutagenicity. A rational sequential screening procedure can improve efficiency and reduce costs when a large number of chemicals is to be tested. Further analyses of the NTP Salmonella database may help improve testing strategies, and also have the possibility of leading to a more rational use of Salmonella mutagenesis for predicting carcinogenicity.

PUBLICATIONS

Zeiger, E., Risko, K.J., and Margolin, B.H.: Strategies for mutagenicity screening using the Salmonella/microsome mutagenicity test. Environ. Mutag., in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21039-01 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Control of Sister Chromatid Exchange in Yeast

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: M.A. Resnick Research Geneticist

CGTB

NIEHS

Others: A.M. Chaudhury Visiting Fellow

CGTB

NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

1.0

1.0

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In an attempt to understand the genetic control and molecular mechanisms of sister chromatid exchange in the yeast *Saccharomyces cerevisiae*, forty-three putative mutants with elevated levels of sister chromatid exchange have been isolated. These mutants were isolated in a strain that is deleted for the resident HIS3 gene and that contains two truncated copies of the HIS3 genes integrated near the centromere of chromosome IV; unequal sister chromatid exchange between homologous regions of the HIS3 gene can restore the functional gene. Mutants elevated for sister chromatid exchange were identified based on their increased ability to form HIS⁺ prototrophs compared to the control unmutagenized clones. Experiments are underway to characterize these mutants in terms of general effects on recombination, DNA damage induced events, mutagenesis and molecular/enzymological defects.

PROJECT DESCRIPTION

METHODS EMPLOYED: A strain of yeast, *Saccharomyces cerevisiae*, that contains two fragments of the *HIS3* gene integrated near the centromere of chromosome IV was used to obtain mutants with altered levels of sister strand exchange. In this strain, stable *HIS*⁺ colonies can be formed only by unequal sister strand exchange.

Yeast strains with the above construction were mutagenized with EMS. Surviving colonies were then grown on rich medium at 25°C for five days. About 30,000 colonies were replica-plated on histidine omission media, incubated at 36°C, 30°C and 25°C, and screened for their ability to form a higher number of *HIS*⁺ prototrophs than the unmutagenized parent.

MAJOR FINDINGS AND PROPOSED COURSE: A variety of interactions between DNA molecules occurs in a living cell. Recombination between homologous chromosomes, which is important for generating genetic diversity, has generally been studied genetically in terms of the production of offspring with novel genotypes, i.e., with a phenotype dissimilar from either parent. Using this criterion, genes have been identified whose products mediate the process of genetic recombination between homologs, thus providing an opportunity to eventually identify and characterize all the factors required for homologous recombination. On the other hand, very little is known about genetic control of exchanges between sister chromatids. It is known that the process occurs in higher and lower eukaryotes. A high incidence of sister chromatid exchange has been observed in cells from patients with Bloom's syndrome, a hereditary human disease, and following treatment with DNA damaging agents. Unequal sister strand exchange has also been postulated to be the mechanism that alters the copy number of genes. Thus, it is important to understand the genetic control of this process.

Since equal exchanges between sister DNA strands does not produce novel genotypes, such events are genetically silent. However, unequal sister strand exchange between two truncated copies of a gene can be studied; such events will restore a functional gene that can be followed phenotypically. Recently a yeast strain has been constructed (M. Fasullo, Stanford) which allows specific detection of unequal sister strand exchange; in this strain, such events generate a *HIS*⁺ colony from among cells which contain duplicated copies of a truncated *his3* gene integrated near the centromere of chromosome IV.

Using the above procedure, we identified 98 initial candidates that appeared to have an increased frequency of formation of *HIS*⁺ colonies. This is the first isolation in any genetically well-defined organism of mutants that are altered in sister chromatid exchange. Colonies of these candidates were purified twice on rich medium at 25°C and tested again for their ability to generate *HIS*⁺ colonies at a high frequency. Forty-three candidates were obtained that generate reproducibly high frequency of *HIS*⁺ colonies compared to control. These 43 candidates fall into the following phenotypic groups: 1) Thirty-two candidates exhibit high frequencies of *HIS*⁺ colonies at all three temperatures tested; 2) Six candidates show a temperature effect; i.e., they produce more *HIS*⁺ colonies at 36°C than they do at 25°C or 30°C.; and 3) Five candidates that show a high frequency of *HIS*⁺ prototrophs cannot grow at 36°C. Some of these candidates show dumbbell shaped cells at 36°C reminiscent of some cell division cycle mutants of yeast.

We plan to characterize the genetic lesions of these putative mutants in the following ways:

- 1) We will determine whether the mutations that enable cells to produce high frequency of HIS^+ prototrophs segregate in a simple Mendelian fashion, i.e., 2:2 segregation.
- 2) We will map these mutations to see if they are allelic to previously isolated mutations in yeast.
- 3) We will determine if the mutations are pleiotropic, especially with respect to other genetic endpoints such as gene conversion, reciprocal recombination, chromosome loss, and mutation frequency.
- 4) Finally, we will determine the physiological and biochemical defects in these mutants. For instance, they might be impaired in cellular functions that are required for DNA metabolism; absence of such function may leave recombinogenic lesions in DNA. Alternatively, they may be derepressed for nucleases that mediate rate limiting steps of DNA-DNA exchanges. This system provides the opportunity to specifically isolate a unique category DNA metabolic mutant.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study provides, for the first time, a tractable genetic means to study the mechanism of sister chromatid exchange. Although sister chromatid exchange in mammalian cells is used routinely to identify genotoxic agents, the mechanism of this process as well as its relationship with other genetic endpoints remains poorly understood. The genetic/biochemical approach described here will not only provide an opportunity to understand the SCE mechanism, but it will also provide a means for determining the relation of SCE to other DNA metabolic events and genetic endpoints.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21045-03 CGTB								
PERIOD COVERED October 1, 1984 to September 30, 1985										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Analysis of <u>SP011</u> , a Gene Required for the Early Events of Meiosis in Yeast										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.: C.N. Giroux</td> <td style="width: 33%;">Senior Staff Fellow</td> <td style="width: 33%;">CGTB</td> <td style="width: 33%;">NIEHS</td> </tr> <tr> <td>Others: H.F. Tiano</td> <td>Biologist</td> <td>CGTB</td> <td>NIEHS</td> </tr> </table>			P.I.: C.N. Giroux	Senior Staff Fellow	CGTB	NIEHS	Others: H.F. Tiano	Biologist	CGTB	NIEHS
P.I.: C.N. Giroux	Senior Staff Fellow	CGTB	NIEHS							
Others: H.F. Tiano	Biologist	CGTB	NIEHS							
COOPERATING UNITS (if any)										
LAB/BRANCH Cellular and Genetic Toxicology Branch										
SECTION										
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709										
TOTAL MAN-YEARS: 1.4	PROFESSIONAL: 0.5	OTHER: 0.9								
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews										
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The goal of this project is to identify and analyze the cellular functions which are required specifically for <u>meiosis</u> in the <u>yeast</u>, <u>Saccharomyces cerevisiae</u>. In particular, we are focusing on the analysis of the <u>SP011</u> gene of yeast which is required for <u>recombination</u> and proper chromosome segregation during meiosis. A general system has been developed to isolate meiosis specific genes of yeast for which mutants are available. Using this system, the <u>SP011⁺</u> wild type gene has been isolated following <u>transformation</u> and <u>complementation</u> of a <u>spoll-1</u> mutant with a total genome clone bank. The function of the cloned gene has been examined during a detailed analysis of the complementation of the <u>spoll-1</u> mutant by the isolated <u>SP011⁺</u> gene. The structure of the cloned gene has been partially determined by restriction enzyme analysis; it is being further analyzed by fine structure restriction analysis and by subcloning. The DNA sequence of the cloned <u>SP011⁺</u> gene will be determined. The function of the cloned gene will be further characterized by <u>mutagenesis</u> of the cloned DNA and by substitution of the chromosomal <u>SP011⁺</u> gene by <u>in vitro</u> engineered constructions. We will try to identify the gene product of the <u>SP011⁺</u> gene by expression of the cloned gene in an <u>E. coli</u> system. </p>										

PROJECT DESCRIPTION

METHODS EMPLOYED: Meiosis mutants of Saccharomyces cerevisiae are grown, sporulated, and recombination frequencies are determined using standard yeast procedures. Cloned DNA sequences are examined for complementation in meiosis by sporulating mutant strains following transformation by, and selection for, the cloned DNA inserted into an E. coli - yeast plasmid shuttle vector. Manipulations of the cloned DNA are performed using standard recombinant DNA procedures. Genetic mapping is performed using tetrad analysis.

MAJOR FINDINGS AND PROPOSED COURSE: Eukaryotes have developed meiosis as a universal and highly structured mechanism to insure the faithful transmission of genetic information to the next generation. Because they are the carriers of genetic information, an understanding of the behavior of chromosomes and their associated DNA metabolism during these unique meiotic events is critical to an evaluation of the genetic consequences of environmental challenges to germ line cells.

The strategy adopted to investigate these problems is to focus on the cellular functions which are specific, or most prominently required, for the unique metabolism of chromosomes during meiosis in the yeast, Saccharomyces cerevisiae. A three-step experimental approach is employed: 1) identify meiosis-essential functions by mutation; 2) isolate the meiotic functions by gene cloning; and 3) analyze the function of the cloned gene by in vitro mutagenesis, gene replacement in yeast, and identification of the gene product in E. coli by recombinant DNA procedures.

The SP011 gene is required for the completion of meiosis and sporulation in Saccharomyces. The spoll-1 mutant is defective in the early events of meiosis; it fails to undergo meiotic recombination and exhibits extensive aneuploidy following the first reductional chromosome segregation. Unlike other recombination-deficient meiotic mutants, however, spoll-1 is not repair-deficient and does exhibit normal levels of mitotic recombination. To analyze the function and regulation of this meiosis-specific gene, a genetic system has been devised to allow its physical isolation by cloning, following transformation and complementation of the spoll-1 mutation during meiosis. Using this system, a clone bank has been screened and a complementing clone has been isolated. This complementing clone has been demonstrated to be the wild type SP011 gene. The identity of the SP011 clone was verified by genetic mapping following re-integration of the cloned DNA by homology at the SP011 locus on chromosome VIII. The precise nature of the complementation by the cloned gene was characterized. Quantitative complementation of all three of the meiotic defects of the spoll-1 mutant has been demonstrated: 1) wild type sporulation frequencies and kinetics are observed; 2) meiotic levels of recombination are evident; and 3) viability and correct ploidy of the meiotic products are restored.

Complementation by the SP011 gene is successful using either single copy integrants or a higher copy number autonomously replicating plasmid. Thus, the cloned SP011 gene is functional in both low and high copy number and in either cis (tandem duplication) or trans (extrachromosomal plasmid) configurations with respect to the spoll-1 mutation.

A gross restriction map of the cloned DNA has been established. The structure of the cloned SP011 gene is being further examined by fine-structure restriction mapping. The function of the cloned gene is being analyzed by subcloning and by insertional mutagenesis using the bacterial transposon, Tn5. The function of the cloned gene will be further characterized by mutagenesis of the cloned DNA and by substitution of the chromosomal SP011 gene by in vitro engineered constructions. An attempt will be made to identify the gene product of the SP011 gene by expression of the cloned gene in an E. coli system.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The gene products which mediate meiotic DNA metabolism in germ line cells are at present unidentified. The physical isolation of the SP011 gene represents the first isolation and identification of a gene which is specifically essential for meiosis. This purified gene will be used as a probe of the mechanism and regulation of meiosis in yeast. This fundamental information is necessary for an understanding of the different genetic susceptibilities and properties of somatic versus germ line cells in response to environmental challenges.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21048-02 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of a Molecular System to Study Mutagenesis in Yeast

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: C.N. Giroux

Senior Staff Fellow

CGTB

NIEHS

COOPERATING UNITS (if any)

Dr. Bernard Kunz, Biology Department, York University, Toronto, Ontario

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

0.6

PROFESSIONAL

0.3

OTHER

0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The focus of this project is to investigate the mechanisms whereby genetic information is transmitted to progeny somatic cells with fidelity: how mutagenesis occurs, and what mechanisms the cell employs to avoid mutation. Using a combination of classical genetic and recombinant DNA techniques, we have constructed a model system to examine the molecular basis of mutagenesis in the yeast, Saccharomyces cerevisiae. Construction of a yeast tester strain is nearing completion which will enable the mutagenesis of a cloned SUP4 tRNA suppressor gene to be assayed by direct genetic selection and DNA sequence analysis. In addition, the tester strain will allow the role in mutagenesis of the three genetically defined repair pathways of yeast to be examined using the same target plasmid.

PROJECT DESCRIPTION

METHODS EMPLOYED: Standard methods of yeast strain construction and genetic analysis are being used to construct the host yeast. Standard recombinant DNA techniques have been used to construct the target plasmid. Plasmids are transferred between *E. coli* and yeast by DNA isolation and subsequent transformation. DNA sequencing has been performed by the Sanger dideoxy method from either single stranded phage or directly from double stranded plasmid DNA.

MAJOR FINDINGS AND PROPOSED COURSE: Eukaryotes have developed a set of complementary repair systems to deal with the environmental challenge of mutagenic agents. At present, both the nature of the events which lead to lesions in the DNA and the molecular mechanisms by which cellular repair systems metabolize these potentially mutagenic lesions in exposed DNA remain undetermined. Using a combination of classical genetic and recombinant DNA techniques, a model system to examine the molecular basis of mutagenesis in the yeast, *Saccharomyces cerevisiae*, is being constructed. This system uses a cloned suppressor tRNA gene as a mutagenesis target. Loss of target suppressor function following exposure of the test system to mutagenic challenge can be assessed by positive selection for drug resistance and the simultaneous expression of multiple auxotrophic requirements which are suppressed by the target gene. A spectrum of the molecular changes occurring in the target DNA sequence as a consequence of the mutagenic challenge will then be determined following transfer of the mutagenized target gene to *E. coli* and its subsequent DNA sequence determination. Once the mutagenic spectrum has been determined at the molecular level for a particular treatment in a wild type cell, the effects of specific DNA repair systems will be examined. In this manner, the role of specific cellular repair systems in metabolizing DNA damage will be analyzed.

The test system will involve mutagenesis of a yeast strain containing the cloned SUP4 tRNA suppressor gene of yeast on a single copy centromere plasmid (*E. coli*-yeast shuttle vector). The tester strain, which has been partially constructed in a series of yeast crosses using tetrad analysis, contains several ochre mutations in different genes, conferring multiple auxotrophy. In addition, the tester strain contains an ochre-suppressible canavanine-resistant mutation. It also contains stable mutations in genes allowing selective maintenance of centromere plasmids containing the corresponding wild type gene. The centromere plasmid to be used as a mutagenesis target has been constructed by recombinant DNA techniques. The plasmid has origins of replication for both *E. coli* and yeast, the ampicillin resistance gene (selection in *E. coli*) and the URA3 gene of yeast (selection in yeast). Thus, the plasmid acts as a shuttle vector between yeast and *E. coli*. In addition, the plasmid contains a subcloned SUP4 yeast tRNA ochre suppressor gene flanked by appropriate restriction enzyme sites to facilitate DNA sequencing. The complete nucleotide sequence of the SUP4 target DNA has been determined by Sanger dideoxy sequencing. The SUP4 bearing plasmid suppresses the auxotrophic requirements of the tester strain and renders it canavanine sensitive. Mutations in the SUP4 plasmid borne gene will be detected by direct selection for canavanine resistance of the tester strain (loss of suppression of the ochre can1-100 mutation). Following the identification of mutations in the target gene (after appropriate treatment of the mutagenesis test system), the shuttle-vector will be transferred to *E. coli* for DNA sequence analysis of the mutagenized target gene. In this manner, the mutational spectrum of a chemical or physical treatment will be determined at the

DNA sequence level. A method is being developed which allows direct DNA sequence determination of the mutagenized SUP4 gene directly from the double stranded plasmid without prior subcloning into single stranded phage.

Using cloned genes in each of the three previously identified DNA repair pathways of yeast, deletions will be made in the tester strain which eliminate one specific pathway. The mutagenesis spectrum will then be determined for a chemical or radiation treatment in the absence of each of the three DNA repair pathways and compared to the wild type spectrum. In this manner, the involvement of each repair pathway in the metabolism of potentially mutagenic DNA damage will be examined. Cloned rad52 (recombination or double-strand break repair), rad1 (excision repair), and rad6 (error prone repair) genes will be used to differentially eliminate each specific repair pathway. Initially UV mutagenesis will be used as a test case for the system by comparison to the spontaneous background of mutation. The role of rad1, a gene required for UV excision repair, will then be examined.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: At present, little specific information is available about the molecular nature of mutation in eukaryotes. Development of a test system in yeast which would provide DNA sequence information for particular mutagenesis events would help fill this gap in our knowledge. In addition to providing basic information about the nature and mechanism of mutation in a eukaryote, this test system could also be used to evaluate the mutagenic effects of a chemical or physical agent, both in quantitative (dose response) and qualitative (mutational spectrum) terms. By comparing the mutagenic spectrum of the wild type test system with those of the three specific repair pathway deficient test systems, the role of each pathway in mutation avoidance and the metabolism of potentially mutagenic DNA damage will be examined. This fundamental information will allow a more accurate determination of how a eukaryote responds to mutagenic challenge by specific environmental agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21049-03 CGTB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) DNA Synthesis and Metabolism During Meiosis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	M. A. Resnick	Research Geneticist CGTB NIEHS
	A. Sugino	Visiting Scientist LGM NIEHS
Others:	T. Chow	Visiting Associate CGTB NIEHS
	J. Nitiss	Guest Worker CGTB NIEHS
	J. Westmoreland	Biological Laboratory Technician CGTB NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Cellular and Genetic Toxicology Branch		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Unique DNA metabolic activities have been implicated during <u>meiosis</u> and following exposure of mitotic cells to DNA damaging agents. We have characterized both the DNA and the DNA metabolic enzymes at various times in meiosis in wild type and repair-deficient cells of <u>yeast</u> . DNA <u>polymerases I and II</u> increase by approximately two and three times, respectively, during meiosis shortly before the time of meiotic DNA synthesis and <u>recombination</u> . Low levels of pH 8.0 single-strand deoxyribonuclease activity are observed in cells prior to the beginning of <u>meiosis</u> . The activity increases during meiosis beginning around the time of pre-meiotic DNA synthesis. Of particular interest is a nuclease under the control of the <u>RAD52</u> gene. It increases five to ten-fold in <u>RAD⁺</u> cells and decreases toward the end of meiosis. No activity is detected in <u>rad52</u> cells. It therefore appears that there is a coordinated increase in enzyme systems involved in meiotic DNA synthesis and recombination. We are also investigating signals which might be involved in meiotic DNA metabolic events. Although there is very little <u>methylation</u> of DNA in yeast, it appears that there are sequences which become <u>methyalted</u> or <u>Tose</u> methylation during meiosis. The timing and function of these are being pursued. Antibodies which had been raised against several proteins associated with replication or DNA metabolism will be used to probe DNA metabolic activities during meiosis. Using various mutants and conditions of high meiotic efficiency, it may be possible to determine the roles of these proteins during meiotic replication and recombination.		

PROJECT DESCRIPTION

METHODS EMPLOYED: Standard procedures for growing yeast are being used in this study. We are using specially developed strains in which synchronous meiosis can be obtained. Biochemical analysis of polymerases and nucleases are performed on partially purified crude extracts. Methylated DNA is being examined using restriction enzymes that can discriminate methylated sites. Where needed, transformation of yeast or bacteria is performed according to techniques commonly described in the literature. Various repair deficient mutants such as rad52-1 or rad1-1 are used as needed. Appropriate genetic markers allow us to monitor survival and commitment to genetic events in "pullback" experiments by plating cells to diagnostic media at various times during meiosis.

MAJOR FINDINGS AND PROPOSED COURSE: Meiosis in yeast has been shown to be under the control of several genes. Included among these are genes identified as being required for the repair of DNA damage in mitotic cells. Since several of our studies involve the role of repair genes in meiosis and effects of DNA damage, it is important to characterize the DNA metabolic events that occur during normal meiosis and in various repair deficient strains. These efforts have been facilitated by the use of strains we developed which exhibit efficient and rapid meiosis; over 90% of the cells can be induced to sporulate in a fairly synchronous manner. To follow biochemical events, crude extracts of repair proficient and deficient strains are examined at various times during meiosis for polymerase I and II, Mg^{++} dependent and independent single-strand deoxyribonuclease activity, a RAD52 controlled deoxyribonuclease, and capability of extracts to synthesize DNA.

The DNA polymerase I increases by nearly a factor of two just prior to the onset of the meiotic round of DNA synthesis and polymerase II increases nearly three times at the time of DNA synthesis. The single-strand deoxyribonuclease activity measured at pH 8 is very low at the beginning of meiosis and increases near the time of DNA synthesis and recombination. Along with this increase there is a five to tenfold increase in a nuclease which is under the control of the RAD52 gene. In the late stages of meiosis there is a decrease in this particular nuclease. All these processes are meiotic specific since they do not occur or increase in strains that do not undergo meiosis. In rad52 mutants the meiotic round of DNA synthesis occurs; however, the single-strand deoxyribonuclease we identified as being under RAD52 control does not appear. Other nucleases also appear to be derepressed. Although rad50 mutants are also deficient in meiotic recombination, they differ from rad52 in that they do not exhibit chromosomal interactions. We have also examined these mutants for nucleases during meiosis and have found that (similar to rad52) they have very low levels of the nuclease that crossreacted with antiserum raised against a Neurospora crassa endo-exonuclease. However, unlike rad52, other nuclease activities expressed during meiosis in RAD are also expressed in rad50. From these results we have concluded that there is a coordinated increase in enzymes associated with meiotic DNA synthesis and recombination. The results with the RAD52 controlled nuclease in combination with other properties of RAD52 strains would suggest a role for this nuclease in meiotic DNA metabolism and recombination.

Having defined several meiotic events in terms of DNA synthesis, associated enzymes and recombination, we plan to examine various rad mutants that exhibit defective meiosis for changes in these properties. We also plan to examine

enzymes and proteins associated with DNA or specifically with DNA metabolism using antibodies that have been raised by Dr. Chow to DNA related proteins which have been purified by Dr. Sugino from mitotic cells. These will be used in RAD^+ and various rad mutants to examine their function in meiosis and recombination.

One of the possible mechanisms of control of meiotic events is through methylation of DNA. The amount of methylation in yeast is extremely low and it can only be detected with probes and restriction enzymes that recognize differences in methylation. Recent work by Sugino has suggested a role for methylation in DNA synthesis in yeast; it has also been shown in yeast that azacytidine, which inhibits methylation in other systems, greatly increases mitotic recombination. We are utilizing probes that hybridize to various sequences that can be methylated in vivo and examining methylation during meiosis. We plan to utilize several probes for regions involved in DNA synthesis to determine possible controls on meiotic DNA synthesis. Since rad50 mutants do not exhibit meiotic recombination, it will be of interest to see whether some methylation sites respond differently in rad50 as compared to RAD^+ during incubation in meiotic medium.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is now clear based on genetic studies from several organisms that some mitotically identified repair functions are required for meiosis. In addition, there are undoubtedly several meiotic-specific enzyme systems that are involved in the processing of DNA. We are developing an integrated biochemical/genetic approach to understanding DNA metabolic events during the meiotic stage of development. This work with yeast will serve as a model for understanding events in germinal cells of higher organisms.

PUBLICATIONS

Resnick, M.A., Sugino, A., Nitiss, J., and Chow, T.: DNA polymerases, deoxyribonucleases and recombination during meiosis in yeast. Molec. Cell. Biol. 4: 2811-2817, 1984.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21051-02 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cytogenetic Analysis of Mutagen-Sensitive Mutants

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: James M. Mason, Ph.D. Geneticist CGTB NIEHS
Others: Akihiko H. Yamamoto, Ph.D. Visiting Fellow CGTB NIEHS

COOPERATING UNITS (if any)

Department of Genetics, University of California, Davis
Albert Einstein College of Medicine, Bronx, New York

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

1.3

PROFESSIONAL

1.1

OTHER

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mutagen-sensitive mutants defective in DNA repair mechanisms have been collected in Drosophila melanogaster and characterized cytogenetically in order to gain a basic understanding of the genetic control of sensitivity to mutagenic agents. The tests used in the initial characterization of these mutants include genetic and cytogenetic mapping, complementation analysis, tests for sensitivity to unrelated mutagens, and tests for pleiotropic effects on related functions such as recombination. A genetic fine structure map of the mei-41 region has been constructed using several independently isolated alleles. This map confirms the large size of mei-41 found during mutational analysis. The mei-41 locus is estimated to cover approximately 300 kilobase pairs of DNA. A nuclease has been identified and is being purified that is under the genetic control of mei-41. This nuclease is antigenically related to nucleases from yeast and Neurospora.

PROJECT DESCRIPTION

METHODS EMPLOYED: Standard genetic manipulations utilizing well-characterized X-linked mutants and chromosomal aberrations in Drosophila melanogaster are employed. Because the mutagen-sensitive (mus) mutants are X-linked, the presence of these mutants is monitored by mating mus males to attached-X females, treating the progeny with MMS (or other mutagen), and checking the sex ratio of the survivors. Transposon tagging is being used to clone mei-41.

MAJOR FINDINGS AND PROPOSED COURSE: A fine structure map of a portion of the X chromosome has been constructed to clarify the allelic relationships between mutants at two putative mus loci, mus(1)104 and mei-41. The results so far lead to the following conclusions. (a) Two mus(1)104 alleles map within the mei-41 locus and thus are allelic to mei-41. (b) Since mei-41 and mus(1)104 have different effects on meiosis but the same effect on sensitivity to mutagens, it is possible to uncouple the effects of mutants of this locus in different tissues. The reason for this uncoupling may become evident after other alleles are added to the map of this region. (c) The mei-41 locus is very large in recombinational terms (0.4 centimorgans). It is the largest locus known in Drosophila, about 50X the size of a simple gene such as ry. This is consistent with the observation that the mutation frequency at the mei-41 locus is about 25X that of a typical mus X-linked gene. Making the usual assumptions as to the genome size and the amount of recombination in Drosophila, we calculate that the mei-41 locus contains about 300 kb. To confirm that the mei-41 locus is large at the DNA level and to gain some insight into the structure and control of a large locus, new mei-41 mutations are being made using transposons and the locus will be cloned using the transposon tagging method. Sixteen such MMS sensitive mutants have been isolated, including 3 new alleles of mei-41 and 2 new alleles of mei-9. These will be used as markers during the cloning to identify the mei-41 and mei-9 regions. They will also be used to align the restriction map of mei-41 with the genetic map.

A nuclease has been identified and is being purified that is under the genetic control of mei-41. This nuclease is antigenically related to nucleases in yeast and Neurospora.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: An understanding of the action of genes controlling mutagen sensitivity is necessary for understanding DNA repair, mutagenesis, recombination, and chromosome stability.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21052-03 CGTB																				
PERIOD COVERED October 1, 1984 to September 30, 1985																						
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Metabolism of Xenobiotics to Mutagens Using Non-hepatic Microsomal Enzyme Systems																						
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"><tr><td style="width: 30%;">P.I.:</td><td style="width: 30%;">Errol Zeiger</td><td style="width: 30%;">Supervisory Microbiologist</td><td style="width: 10%;">CGTB</td><td style="width: 10%;">NIEHS</td></tr><tr><td>Others:</td><td>D. Pagano</td><td>Microbiologist</td><td>CGTB</td><td>NIEHS</td></tr><tr><td></td><td>T. Eling</td><td>Head, Prostaglandin Group</td><td>LFPT</td><td>NIEHS</td></tr><tr><td></td><td>T. Petry</td><td>Staff Fellow</td><td>LPFT</td><td>NIEHS</td></tr></table>			P.I.:	Errol Zeiger	Supervisory Microbiologist	CGTB	NIEHS	Others:	D. Pagano	Microbiologist	CGTB	NIEHS		T. Eling	Head, Prostaglandin Group	LFPT	NIEHS		T. Petry	Staff Fellow	LPFT	NIEHS
P.I.:	Errol Zeiger	Supervisory Microbiologist	CGTB	NIEHS																		
Others:	D. Pagano	Microbiologist	CGTB	NIEHS																		
	T. Eling	Head, Prostaglandin Group	LFPT	NIEHS																		
	T. Petry	Staff Fellow	LPFT	NIEHS																		
COOPERATING UNITS (if any) Laboratory of Pulmonary Function and Toxicology																						
LAB/BRANCH Cellular and Genetic Toxicology Branch																						
SECTION																						
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709																						
TOTAL MAN-YEARS 0.2	PROFESSIONAL 0.2	OTHER																				
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"><tr><td><input type="checkbox"/> (a) Human subjects</td><td><input type="checkbox"/> (b) Human tissues</td><td><input checked="" type="checkbox"/> (c) Neither</td></tr><tr><td><input type="checkbox"/> (a1) Minors</td><td></td><td></td></tr><tr><td><input type="checkbox"/> (a2) Interviews</td><td></td><td></td></tr></table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews													
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither																				
<input type="checkbox"/> (a1) Minors																						
<input type="checkbox"/> (a2) Interviews																						
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided) <p>Xenobiotic chemicals can be metabolized by organs and tissues other than the liver. Additionally, the <u>prostaglandin endoperoxide synthetase (PES)</u> system is found in a number of organs and is not dependent on <u>cytochrome P-450</u>. The <u>metabolism of aromatic amines, imidazoquinolines and other chemicals to mutagens in Salmonella tester strains</u> was studied using PES. Specifically, the <u>aromatic amines and imidazoquinolines</u> being studied are those formed in beef during cooking, or from amino acids undergoing pyrolysis. All these chemicals are mutagenic following metabolism by liver, and other organ P-450-dependent mixed function oxidases.</p>																						

PROJECT DESCRIPTION

METHODS EMPLOYED: PES metabolism: A crude microsomal preparation containing prostaglandin endoperoxide synthetase is prepared from ram seminal vesicles. This preparation is included in a pre-incubation modification of the standard Salmonella plate test. 9000xg homogenates or purified microsomal preparations are made from other organs using standard techniques. Other: Homogenates are prepared from lungs and kidneys using a standard protocol.

MAJOR FINDINGS: Studies were performed to extend and further define our findings that aromatic amines were metabolized to mutagenic products by prostaglandin endoperoxide synthetase (PES). Using Salmonella strain TA98, preliminary studies on two cooked food mutagens, Trp-P-1 and Trp-P-2, have shown them to be mutagenic in the presence of PES but at much lower response levels than in the presence of rat liver S9. The Trp-P-1 response occurs at a dose approximately ten times lower than the Trp-P-2 response with the Trp-P-2 response peaking at 100 nmoles/pl. The mutagenicity of both compounds is arachidonic acid-independent, implying that the Salmonella may be supplying an endogenous substrate for the PES co-oxidation reaction. Further work in the area of enzyme and substrate inhibition is continuing.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Most current research on the activation of xenobiotics is with liver cytochrome P-450 systems, although other organs and tissues also have the ability to metabolically activate xenobiotic chemicals. Prostaglandin endoperoxide synthetase is ubiquitous throughout mammalian tissues and frequently co-exists with cytochrome P-450, and there is increasing evidence that PES may serve as an alternative or complementary activation system in vivo. This work will help to further elucidate the role of PES and other organs in the metabolic activation of xenobiotics.

PUBLICATIONS

Boyd, J.A., Zeiger, E., and Eling, T.E.: The prostaglandin H synthetase-dependent activation of 2-aminofluorene to products mutagenic to S. typhimurium strains TA98 and TA98NR. Mutation Res., in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21053-02 CGTB

PERIOD COVERED

October 1, 1984 to September 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Genetic Control of Mutation in Drosophila

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	James M. Mason	Geneticist	CGTB	NIEHS
Others:	Larry Champion	Biological Lab Technician	CGTB	NIEHS
	Barry Margolin	Mathematical Statistician	BRAP	NIEHS

COOPERATING UNITS (if any)

Department of Biological Science, Purdue University
Department of Genetics, University of California at Davis
Department of Biology, Brown University
~~School of Biological Sciences, University of Kentucky~~
Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

1.4

PROFESSIONAL

0.4

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mutation rates are under genetic control. In bacteria and yeast, the frequency of induced mutations can be either increased or decreased by blocking one or another pathway of DNA repair. This project is designed to determine the relationship between DNA repair and mutagenesis in Drosophila melanogaster. Three approaches are being taken: (1) A mutant which increases the mutation frequency (a mutator) has been identified, mapped, and characterized. This mutator blocks repair of chromosome breaks specifically in oocytes, thereby allowing a previously undescribed repair process to be observed. In this process broken chromosomes are "healed", allowing the recovery of terminal deletions. (2) The interaction of DNA repair-defective mutants and transposable elements has been observed in double mutant combinations. None of the repair-defective mutants examined to date influence the rates of transposon-induced mutation or recombination, although mutants at the mei-41 locus prevent the transmission of transposon-bearing chromosomes. (3) Aneuploidy is being examined as a genetic endpoint. Chemicals that induce aneuploidy are being identified as probes to investigate mitosis and meiosis.

PROJECT DESCRIPTION

METHODS EMPLOYED: Standard genetic manipulations utilizing well-characterized mutants and chromosomal aberrations in Drosophila melanogaster are employed.

MAJOR FINDINGS AND PROPOSED COURSE: One mutator being examined is unable to repair X-ray induced chromosome breaks in the normal way. In its presence broken chromosomes are recovered which do not appear to be capped by a previously existing telomere; that is, terminal deletions are recovered that are not associated with chromosome rearrangements such as translocations. The mutator is recessive and maps near the end of the left arm of chromosome III. It is active primarily in later stages of oocyte development, but not during spermiogenesis. The mutator shows a slight decrease in the frequency of meiotic recombination but has little or no effect on nondisjunction. It does, however, increase the frequency of X-chromosome loss. Many of these losses are the result of whole arm deletions in which the centromere is recovered but the rest of the X has been lost. The breaks that are recoverable as terminal deletions appear to be randomly distributed in the euchromatin; they can be on any of the four chromosomes and they can be recovered near the tip or in the middle of the X chromosome. Even at the DNA sequence level, breaks are recovered in two different regions with expected frequencies based on the assumption of randomness, and the position of each breakpoint is unique. The telomeres of a number of terminally-deleted chromosomes are being cloned in order to characterize them at the molecular level and develop a consensus sequence for functional telomeres in *Drosophila*. The mutator appears to be able to distinguish euchromatic from heterochromatic breaks because heterochromatic breaks are recovered as multiple break rearrangements, i.e., translocation rather than terminal deletions.

Transposable elements in *Drosophila* cause a wide range of genetic effects including mutation, mitotic recombination, chromosome aberrations, and sterility. These effects have been termed "hybrid dysgenesis". The control of transposition is poorly understood in any system. We have asked whether cellular functions are important in the control of transposition by combining mutants defective in DNA repair and recombination with transposable elements. If such functions are important for transposition, the transposon-induced frequencies of mutation and recombination should be altered; they are not. Mutants at four different loci had no effect on either mutation or recombination frequencies. Mutants at the *mei-41* locus, however, drastically reduced the recovery of transposon-bearing chromosomes in the following generation. The mechanism for this is unknown, but loss of transposon-bearing chromosomes is correlated with dominant lethality.

Malsegregation of chromosomes during mitosis and meiosis is a type of mutation event that is not well understood. We are beginning to examine aneuploidy as a mutational endpoint. Chemicals that induce aneuploidy are being identified as potential probes in the investigation of chromosome segregation. This investigation began with a thorough literature search to identify possible chemical probes and potential target sites.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These studies will lead to an understanding of the cellular mechanisms used to regulate the rates of mutation, transposition, chromosome breakage, and malsegregation in eukaryotic organisms. It should also, in the long run, allow one to sequence

newly-formed telomeres and thereby obtain additional information about the organization and stability of eukaryotic chromosomes.

PUBLICATIONS

Slatko, B.E., Mason, J.M., and Woodruff, R.C.: The DNA transposition system of hybrid dysgenesis in *Drosophila melanogaster* can function despite defects in host DNA repair enzymes. Genet. Res. (Camb.) 43: 159-171, 1984.

Mason, J.M., Strobel, E., and Green, M.M.: *mu-2*: A mutator gene in *Drosophila* that potentiates the induction of terminal deficiencies. Proc. Natl. Acad. Sci. USA 81: 6090-6094, 1984.

Zimmering, S., Mason, J.M., and Osgood, C.: Current status of aneuploidy testing in *Drosophila*. Mutat. Res., in press.

Mason, J.M., and Resnick, M.A.: Mechanisms and detection of chromosome malsegregation using *Drosophila* and the yeast *Saccharomyces cerevisiae*. In Vaughn-Dellarco, V., Voytek, P., and Hollaender, A. (Eds.): Aneuploidy: Etiology and Mechanisms. New York, Plenum Press, in press.

Mason, J.M., Valencia, R., Woodruff, R.C., and Zimmering, S.: Genetic drift and seasonal variation in spontaneous mutation frequencies in *Drosophila*. Environ. Mutagen., 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21054-02 CGTB									
PERIOD COVERED October 1, 1984 to September 30, 1985											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) DNA Damage and Repair in Centromeres of Yeast											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.: M. A. Resnick</td> <td style="width: 33%;">Research Geneticist</td> <td style="width: 15%;">CGTB</td> <td style="width: 19%;">NIEHS</td> </tr> <tr> <td>Others: J. Westmoreland</td> <td>Biological Laboratory Technician</td> <td>CGTB</td> <td>NIEHS</td> </tr> </table>			P.I.: M. A. Resnick	Research Geneticist	CGTB	NIEHS	Others: J. Westmoreland	Biological Laboratory Technician	CGTB	NIEHS	
P.I.: M. A. Resnick	Research Geneticist	CGTB	NIEHS								
Others: J. Westmoreland	Biological Laboratory Technician	CGTB	NIEHS								
COOPERATING UNITS (if any) Dr. Kerry Bloom, Assistant Professor, University of North Carolina, Chapel Hill											
LAB/BRANCH Cellular and Genetic Toxicology Branch											
SECTION											
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709											
TOTAL MAN-YEARS: 0.7	PROFESSIONAL: 0.2	OTHER: 0.5									
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews		
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<input type="checkbox"/> (a1) Minors											
<input type="checkbox"/> (a2) Interviews											
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The induction of <u>DNA damage</u> in chromosomal DNA would be expected to be dependent on the total structure of <u>chromosomes</u> within cells. Protein associations, folding, and extent of <u>superhelicity</u> of DNA within chromosomes could influence the induction and distribution of damage. Because of the role that <u>centromeres</u> play in chromosome <u>segregation</u>, we have begun to examine the distribution of damage in the centromeric region of cellular DNA. Excision-defective strains of <u>yeast</u> are irradiated with UV, the DNA is gently extracted and treated with UV-endonuclease to produce nicks next to <u>pyrimidine dimer</u> sites. The chromosomal DNA is then probed with a probe specific for the centromere of chromosome III. Compared to DNA irradiated in vitro, the centromere sequence of DNA from cells irradiated in vitro is less sensitive to UV and shows a different pattern of UV-sensitivity. These results suggest that the centromere-associated proteins may influence sensitivity. There is a significant amount of lesions on opposite strands that are sufficiently close so as to lead to double-strand breaks when the DNA is treated with the UV-endonuclease. Both single-strand and double-strand damage occur at sufficiently high frequencies in the centromere region to implicate them as potential inducers of chromosome loss. </p>											

PROJECT DESCRIPTION

METHODS EMPLOYED: Excision-defective strains of the yeast *Saccharomyces cerevisiae* are being used in the initial phases of the experiments wherein we are attempting to determine the distribution of UV induced pyrimidine dimers. Cells are grown to log phase under standard conditions. Following UV irradiation the cells are lysed gently and extracted with phenol. The large molecular DNA that is recovered is restricted with enzymes: a) so that subsequent handling will not introduce breaks, and b) to enable detection of unique fragments. The DNA is then treated with our preparation of a UV endonuclease which is specific for pyrimidine dimers and the DNA is run on gels. The DNA is transferred to nitrocellulose filters and probed with the appropriate centromere and chromosomal probes. Dimers in the centromeric region are revealed by a shift in the probed position when the DNA is treated with the UV endonuclease as compared to no treatment.

MAJOR FINDINGS AND PROPOSED COURSE: Specifically, we plan to characterize the distribution of UV-induced pyrimidine dimers in the centromere III region of yeast and relate this to the chromosomal structure in this region, since the distribution of nucleosomes and centromeric protein(s) are known. Having done this, we then plan to examine the repair of dimers in this region and compare the repair efficiency to other regions of the genome.

Procedures have been developed for the routine isolation of large molecular weight DNA which lacks single strand breaks. Following the restriction of this with the appropriate enzymes, the background is extremely low which allows for quantitation of a relatively small amount of damage. Only DNA which has been UV-irradiated is nicked by the UV-endonuclease. Experiments to date have been designed to address the following issues: sensitivity of DNA to UV when it is irradiated in the cells versus *in vitro*; influence of chromatin and centromeric protein on susceptibility of cellular DNA to UV; sensitivity of the centromere essential region; induction of double-strand events.

There appears to be no general difference in sensitivity for DNA which is part of chromatin in cells as compared to DNA which has been purified. However, differences have been observed in the pattern of pyrimidine dimer induction suggesting an influence of chromosome structure on the induction of DNA damage. The centromere region is particularly sensitive; this is presumably due to the high AT content. The pattern is modified by the presence of centromere protein in the cell and the induction in this region appears to be somewhat less, suggesting possible protection. This is most noticeable for DNA damage on opposite strands which is sufficiently close (about 10 base pairs) such that UV-endonuclease cuts will result in double-strand breaks. There is a significant difference in the pattern of double strand damage between *in vivo* and *in vitro* DNA. This further supports the idea that a chromosome may have a unique structure that may affect sensitivity to DNA-damaging agents.

Experiments are being directed to quantitatively evaluate the induction of pyrimidine dimers in relation to the distribution of chromosome proteins. The specific role of the centromere proteins will be evaluated using plasmids in which the cloned centromere regions can be turned on or turned off in terms of associated centromere proteins. Repair of damage in the centromere and surrounding regions will be examined in terms of single-strand and double-strand damage. It appears

that double-strand damage is not efficiently repaired, implicating this type of damage as having an important biological effect on cells, particularly in the centromere region. Single-strand damage is rapidly repaired throughout the centromere region. We are also evaluating the effects of UV and other damaging agents on the induction of aneuploidy. It may be possible to correlate damage in the centromere with chromosome instability.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: To understand how various external chemical agents affect the genetic makeup of an organism, it is important to determine the interactions of the agents with chromosomal DNA. The interactions and the nature of the DNA damage resulting from treatment with a particular agent could be greatly affected by the complex structure of chromosomes and chromosomal DNA. One of the most important functional structures in the chromosome is the centromere. Our approach to specifically characterizing damage and DNA repair in this region should advance our understanding of the interactions between DNA damaging agents and chromosomal structures and aid us in understanding the events which lead to aneuploidy.

The distribution of damage from various agents in chromosomal DNA would be expected to influence the genetic consequences of that damage. Chromosome structure would be expected to affect distribution. Probably the single most important structure in chromosomes is the centromere, since it is required for proper chromosomal assortment. While it is known that damaging agents can lead to malsegregation of chromosomes, there is no information about the distribution of damage in centromeric DNA, the effects of damage at the centromere, or even whether repair can take place. Because of the recent isolation of centromeric sequences from yeast, these issues can now be approached.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21058-03 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Molecular Dosimetry of Ethylmethane Sulfonate in Salmonella, Low-Dose Mutagenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: E. Zeiger

Supervisory Microbiologist CGTB

NIEHS

COOPERATING UNITS (if any)

Z. Matijasevic, Lab. of Biology and Microbial Genetics, Univ. Zagreb, Yugoslavia

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

0.1

PROFESSIONAL

0.1

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mutagenic activity of ethylmethane sulfonate (EMS) as a function of its DNA alkylating ability has been studied in *Salmonella typhimurium*. The mutagenic activity of EMS in the base-pair substitution strain G46 and its repair deficient derivatives (TA1950, [uvrB]; TA92, [pKM101]; TA2410, [uvrB, pKM101]) were compared. Ethylation levels were equivalent in wild type and uvrB cells, but the efficiency of induction of mutations (mutants/adduct) was different between the two cell types. EMS and other ethylating agents induce higher levels of mutation in wild type cells at low doses; at high doses the uvrB⁻ cells show greater incidences of mutation. This phenomenon is being studied with other classes of alkylating agents and in other microbial strains.

PROJECT DESCRIPTION

METHODS EMPLOYED: Mutagenicity determinations are done by standard suspension assays.

MAJOR FINDINGS AND PROPOSED COURSE: EMS-induced reversion frequencies of the four *Salmonella* strains increased as a function of dose and the level of DNA ethylation was proportional to the administered dose. Comparison of *uvrB*⁻ with wild type strains showed that all strains contained equivalent numbers of DNA adducts. Two types of mutagenic responses were seen. At EMS doses of 10 µg/ml and greater, higher levels of mutation were obtained in the *uvrB*⁻ than in wild-type cells, as expected. However, in the dose range of 2-5 µg EMS/ml, the wild-type cells responded with a higher frequency of mutations than the *uvrB*⁻ cells; the pKM101 plasmid did not eliminate this effect. Experiments with other ethylating agents and with MNNG have shown that this low-dose effect is not confined to EMS. Studies are underway with isogenic strains of *E. coli* to determine whether the effect seen with *Salmonella* (which contains a partially-defined *uvrB* deletion) can be replicated in a strain containing a defined mutation in the *uvr* locus. Further research plans include the isolation of a better-defined *uvrB*⁻ strain of *Salmonella* and the use of other types of alkylating agents to determine whether the low dose effect is also seen with other types of DNA adducts.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The estimation of risk for mutagenic agents involves knowing not only the amount of chemical the target cell is exposed to, but the delivered dose as well. That is, the amount of active mutagen that interacts with the target molecule, DNA, to form a premutagenic lesion. This study will provide such data using the standard mutagen, EMS, and the results can be compared with results from similar studies using other bacteria, yeast, cultured mammalian cells, and mammalian tissues *in vivo*. This will determine the degree in which mutagenicity results from microbial cells can be extrapolated to mammalian cells. The low-dose mutagenesis data imply that dose responses from high dose mutagenicity experiments cannot be extrapolated to low dose exposures. The proposed studies will define low-dose mutagenic responses for a number of model mutagens and environmental chemical mutagens in order to determine the validity of extrapolating from high to low dose.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 60102-07 CGTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Testing of Chemicals of Interest in Salmonella

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Errol Zeiger	Supervisory Microbiologist	CGTB	NIEHS
Others:	D. Pagano	Microbiologist	CGTB	NIEHS

COOPERATING UNITS (if any)

International Program on Chemical Safety, World Health Organization
Avishay A. Stark, Department of Biochemistry, Tel Aviv University, Israel

LAB/BRANCH

Cellular and Genetic Toxicology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

0.5

PROFESSIONAL

0.4

OTHER

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Chemicals of interest have been tested for mutagenicity using a number of Salmonella tester strains. The chemicals studied, or being studied, were sodium bisulfite (SB), a series of nitrosamines, 2- and 4-acetylaminofluorene (2AAF and 4AAF), benzo(a)pyrene (BaP), pyrene, and a series of N-substituted phenanthrene-imines. SB, an antioxidant, has been shown to be mutagenic at low pH, and the mutagenicity was bacterial strain-specific. The carcinogen-noncarcinogen pairs, 2AAF and 4AAF, and BaP and pyrene, were tested both in vitro and in the intra-sanguineous host-mediated assay (HMA). All 4 chemicals are mutagenic in vitro, and the carcinogens (2AAF and BaP) gave consistently higher responses than the noncarcinogens (4AAF and pyrene). All 4 chemicals failed to induce significant mutagenic responses in the host-mediated assay. The cyclic nitrosamines are being tested in the HMA to determine if their pattern of organ-specific carcinogenicity can be predicted by any organ-specific mutagenicity detected in the HMA. The mutagenicity of the phenanthreneimines correlated with the electron-accepting or -donating ability of the substituent group, and inversely with their alkylating ability.

PROJECT DESCRIPTION

METHODS EMPLOYED: The standard Salmonella plate test with some modifications, a Tiquid, pre-incubation assay, or an intrasanguineous host-mediated assay, were employed with various Salmonella strains. Metabolic activation preparations were derived from a number of sources.

MAJOR FINDINGS AND PROPOSED COURSE: SB was shown to be mutagenic using strain TA97, but only at pH 5 or 6. At pH 7.0 or higher, there was no mutagenicity seen and decreased toxicity was noted. When SB was tested with other Salmonella strains (G46 series), the mutagenic responses obtained were inconsistent. SB was not mutagenic to TA1538 or TA98, strains which, like TA97, are sensitive to frame-shift mutagenesis.

Bisulfite can specifically deaminate cytosine in single strand DNA under conditions of high concentration and acidic pH. Although deamination by bisulfite is a very specific reaction in vitro, it is not known if this reaction occurs in vivo or if it will cause the same specific mutation. Sodium bisulfite was tested in Salmonella strain TA97, since this tester strain has a run of 6 cytosines at the target site in the hisD gene, and produced a reproducible, dose-related increase in mutation. The dose responses were seen at pH 5 and 6 and decreased as the pH was increased to 7, 8, or 9. Toxicity also decreased with increasing pH. All strains of Salmonella containing the hisD6610 mutation show this response, whereas Salmonella strains containing the hisG46 mutation (a run of 3 cytosines at the target) yield weak or equivocal results. Strains containing the hisC3076, hisD3052, or hisG428 mutations are not mutagenized. Neither the plasmid, pKM101, nor the uvrB gene influence the positive responses. We are investigating the possibility that the positive bisulfite response may be due to its metabolism by enzymes of the cysteine biosynthetic pathway in bacteria rather than from any of the known in vitro reactions. In support of this hypothesis are the facts that bisulfite has been shown to be a mutagen in bacteria and plants but not mammalian cells; both bisulfite and cysteine inhibit the mutagenicity of sodium azide, a chemical which is metabolized to a mutagen by enzymes of the non-mammalian, cysteine biosynthetic pathway; and that sodium azide is also mutagenic in only bacterial and plant cell systems. This study may explain how a mutagenic metabolite of bisulfite may arise.

The carcinogens, BaP and 2AAF, and their non-carcinogen analogues, pyrene and 4AAF, were shown to be mutagenic in Salmonella strain TA100 using the standard plate incorporation method in the presence of mouse liver S9 activation. BaP, 2AAF and 4AAF were mutagenic in the presence of uninduced mouse S9, but pyrene required induced S9 for any significant level of mutagenicity to be seen. The peak response for BaP and pyrene in the presence of uninduced mouse S9 were seen at doses between 12-18 $\mu\text{g}/\text{pl}$. At these doses, the BaP response was 21X the control compared with the pyrene response which was 1.3X the control. In the presence of induced rat S9, the BaP response peaked at 18 $\mu\text{g}/\text{pl}$ (30X the control) and the pyrene response at 6 $\mu\text{g}/\text{pl}$ (2.3X the control). 2AAF gave a maximum response at 100 $\mu\text{g}/\text{plate}$ (52X the control). At this dose, 4AAF was only 1.8X the control. The peak response for 4AAF was at 600 $\mu\text{g}/\text{plate}$ and was about 5.5X the control. From these results, it can be seen that the carcinogens produced the higher mutagenic responses and were easily distinguishable from their non-carcinogen analogues. All four of the chemicals failed to induce any significant

mutagenic responses when tested for mutagenicity in the HMA using *Salmonella* strain TA100. These results may be due to the higher spontaneous mutation frequency of this strain in vivo, the poor solubility and/or absorption of these compounds, the large day-to-day and mouse-to-mouse variation that was seen, or to the more efficient detoxifying metabolism of the liver in situ as compared to S9 preparations.

Phenanthreneimine and its N-substituted derivatives were all direct-acting mutagens. N-substitution with electron-releasing groups resulted in chemicals that were more mutagenic than those substituted with electron-withdrawing groups. Except for the parent imine, alkylating activity was inversely correlated with mutagenicity. These relationships will be further studied using other N-substituted phenanthreneimines.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The host-mediated assay (which has been proposed for mutagenicity screening and risk assessment procedures) is being studied to evaluate its role in this process. Preliminary evaluations have suggested that the HMA may not be suited for screening purposes, but more detailed studies are necessary in order to define its role in risk assessment, and under what conditions it can fulfill that role. The presence of a positive mutagenic response in SB under certain pH conditions and in select strains adds to the growing concern that this food additive needs more extensive study. The study with phenanthreneimines is examining the relationships between chemical structure, chemical activity, and mutagenicity. The majority of chemicals now being tested are confined to chemical classes of which the mutagenicities of representative chemicals are already known. It then becomes important to look at chemical class analogues and the many variations possible in order to assess the overall potential hazard of a particular class of chemicals. A study of N-substituted phenanthreneimines and the possible relationships that exist between structure, alkylating ability, and mutagenicity is one such approach to chemical class hazard assessment. These results will enable us to better predict potential mutagenicity from a chemical's structure.

PUBLICATIONS

Matijasevic, Z., and Zeiger, E.: Mutagenicity of pyrene in *Salmonella*. Mutat. Res. 142: 149-152, 1985.

Pagano, D.A. and Zeiger, E.: The stability of mutagenic chemicals stored in solution. Environ. Mutag. 7: 293-302, 1985.

Pagano, D.A., and Zeiger, E.: The mutagenicity of the carcinogens, 2-acetylaminofluorene and benzo(a)pyrene, and their noncarcinogen analogues, 4-acetylaminofluorene and pyrene, using *Salmonella* in the Host-Mediated Assay. In press.

Pagano, D.A. and Zeiger, E.: The mutagenicity of the carcinogens, 2-acetylaminofluorene and benzo(a)pyrene, and their noncarcinogen analogues, 4-acetylaminofluorene and pyrene, using reversion at the histidine locus of *Salmonella typhimurium*. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 60122-06 CGTB						
PERIOD COVERED October 1, 1984 to September 30, 1985								
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Mechanisms of DNA Repair in Yeast and Their Role in Meiosis								
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.: M. Resnick</td> <td style="width: 33%;">Research Geneticist</td> <td style="width: 33%;">CGTB NIEHS</td> </tr> <tr> <td>Others: J. Nitiss</td> <td>Guest Researcher</td> <td>CGTB NIEHS</td> </tr> </table>			P.I.: M. Resnick	Research Geneticist	CGTB NIEHS	Others: J. Nitiss	Guest Researcher	CGTB NIEHS
P.I.: M. Resnick	Research Geneticist	CGTB NIEHS						
Others: J. Nitiss	Guest Researcher	CGTB NIEHS						
COOPERATING UNITS (if any) J. C. Game, University of California, Berkeley, Department of Genetics R. Malone, Loyola University Medical School, Chicago, Illinois R. M. Roth, Illinois Institute of Technology, Chicago, Illinois								
LAB/BRANCH Cellular and Genetic Toxicology Branch								
SECTION								
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709								
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 0.2	OTHER: 1.3						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews								
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> <u>DNA repair systems identified in mitotic cells of the yeast <i>Saccharomyces cerevisiae</i> are being examined for a) their protection of cells undergoing meiosis, and b) the role of the corresponding genes in normal meiosis. We have developed unique sucrose gradient techniques to examine repair after low doses of UV or ionizing radiation and to follow changes in meiotic DNA during meiosis.</u> </p> <p> <u>The RAD50, RAD52 and RAD57 genes are essential in the repair of DNA double-strand breaks in mitotic cells. They are also required for meiosis. Mutations in these genes abolish normal meiotic recombination; it appears that RAD50 acts at an early step in meiosis. Rare single-strand interruptions (SSIs) were observed in rad52 and rad57 strains shortly after the beginning of meiotic DNA synthesis and these appear to be related to recombination. Gentle isolation techniques have allowed the characterization of SSIs as breaks in DNA; many have 3' OH and 5' PO₄ termini. The SSIs do not appear to be randomly distributed, based on experiments involving probes for specific chromosomal regions, suggesting specific sites or regions involved in normal meiotic recombination.</u> </p> <p> <u>While rad52 and rad57 mutants are defective in meiotic recombination, recombinants can be recovered prior to commitment to reductional division. The frequency in rad52 mutants is much lower than in RAD⁺ strains; the frequency is somewhat lower in rad57 mutants. The recombinants are also qualitatively different from those in RAD⁺. When meiosis is arrested and rad52 or rad57 are exposed to growth medium, recombinants are not recovered. This is due to the extended time necessary for recombinants to form, and suggests that rad52 and rad57 are blocked at an intermediate step in recombination. The recombination intermediates are resolved slowly, and growth prior to resolution prevents the appearance of recombinants.</u> </p>								

PROJECT DESCRIPTION

METHODS EMPLOYED: Various repair-deficient mutants of *Saccharomyces cerevisiae* are genetically manipulated and grown using standard methods. Strains have been developed to exhibit a high level of synchronous meiosis. Appropriate genetic markers allow us to monitor survival and commitment to genetic events in "pullback" experiments by plating cells to diagnostic media at various times during meiosis. In this way we can relate DNA damage, repair defects, and normal or damage induced molecular changes in chromosomal DNA to specific events in the meiotic cycle, such as DNA synthesis or commitment to recombination. To examine molecular events, the radioactively-labeled cells are examined for the appearance of breaks using sucrose gradient and gel electrophoresis techniques.

MAJOR FINDINGS AND PROPOSED COURSE: Meiosis is a period in the developmental cycle of eukaryotes during which there is intense genetic activity. We have shown that the products of genes involved with repair in mitotic cells can also be involved in normal meiosis and some repair mutants die during a commitment to meiotic development. These repair systems can be viewed as being mitotically dispensible and meiotically essential. Little is known about the systems available for protecting cells during germinal development or the molecular events which occur during normal meiosis. Our research objectives can be divided into two broad categories. The first is directed toward determining the repair systems available to cells undergoing germinal development and the effects of DNA damage when repair is present or absent. The second objective is to understand the role of genes identified as being involved with repair in the normal meiotic process.

We have found that in mitotic cells the RAD50, RAD52 and RAD57 genes are essential for DNA damage induced recombination, resistance to X-rays and DNA double-strand break repair. These genes are essential for normal meiosis and meiotic recombination. In pullback experiments, the rad50 cells exhibit no commitment to recombination regardless of when they are harvested after introduction into meiotic medium. Although rad50, rad52, and rad57 cells die as they proceed through meiosis, a low level of commitment to recombination does occur in meiosis in rad52 mutants; a high level occurs in rad57 strains. We have concluded that RAD50, RAD52, and RAD57 are essential for interchromosomal recombination during meiosis and we have proposed that accumulated intermediates in rad52 and rad57 mutants may be processed in the pullback experiments so as to yield recombinants.

The recombinants found in rad52 and rad57 strains in pullback experiments are different than those recovered in RAD⁺ strains. If cells are exposed to rich media, recombinants induced by meiosis are not recovered. Only by holding in non-growth conditions for a minimum of 8-12 hours can recombinants be recovered. Thus, the single strand breaks seen during meiosis (see below) may be recombination intermediates that are processed by another pathway. Since rad52 strains are completely unable to repair a double strand break, this recombination probably does not pass through that type of an intermediate.

Having concluded that double-strand break repair involved recombination mechanisms and that the rad50, rad52 and rad57 mutants were defective in mitotic and meiotic recombination, we examined the chromosomal DNA of RAD⁺ and these mutants. Neither RAD⁺ nor rad50 cells exhibit any changes in the size of single- or double-strand DNA during meiosis. Based on other results, the rad50 may be blocked at an early

step in meiosis so that recombinational events are not initiated while in RAD^+ any breaks which might occur could be short-lived. Unlike the wild-type and rad50, the rad52 and rad57 mutants accumulate single-strand interruptions (SSIs) during meiosis; double-strand breaks are not observed. The 200-400 SSIs per cell correlates well with the genetic exchanges in meiotic cells and in combination with other evidence suggests they are important in recombination.

To characterize SSIs further, we have developed a method for the gentle isolation of DNA without the artifactual introduction of breaks. Chromosomal DNA from gently lysed cells is sedimented through sucrose gradients containing a layer of blunt-end cutting restriction enzyme; the restriction fragments can then be handled without concern about breakage and the SSIs should be the major category of internal interruptions within fragments. We have examined the DNA from RAD^+ , rad50 and rad52 throughout meiosis for ability to act as primer in DNA synthesis reactions involving *E. coli* DNA polymerase I. Consistent with sucrose gradient results, the only DNA which serves as primer is that of the rad52 mutant at late times in meiosis. The inability to observe synthesis with T4 polymerase demonstrates that the SSIs are nicks not gaps and the ability to decrease the polymerase I synthesis by prior treatment with ligase suggests that some of them have a 3' OH and 5' PO_4 configuration.

We are examining the locations of SSIs using a technique which we developed for examining individual chromosomes. Fractions from gradients are probed with regions from specific chromosomes; an individual chromosome should show up as a sharp peak. When fractions from alkaline sucrose gradients of meiotic rad52 cells are probed with a SUP4-CDC8 cloned region of chromosome 10 only fractions containing small DNA hybridize. Fractions containing large DNA hybridizes with this probe when meiotic RAD^+ or mitotic cells are used. The nature of the distribution is such that the SSIs that give rise to these fractions could not occur randomly. With rad50 the probe only binds to large DNA, consistent with previous sucrose gradient results. A deoxyribonuclease which we have identified in RAD^+ and is absent in rad52 mutants appears to be important in repair and normal meiosis; possibly it processes the SSIs.

Using strains containing a rad52 null mutation, we will pursue further the role of rad52 in meiosis. Observations with the null mutant in terms of recombination are comparable to those with the previous rad52-1 mutation, which was due to a single base change. We will also examine mutants other than rad50 and rad52 that are associated with mitotic recombinational repair for their role in meiosis.

We are using several probes to examine the generality of region (or site) specific SSIs in rad52 mutants. Included will be sequences that correspond to regions of high genetic recombination. Of particular interest are the regions or sites at which the SSIs appear. To identify these we will develop a series of probes and "walk" to the specific interruptions. Assuming that such regions in plasmids also accumulate SSIs, we plan to reduce the size of the regions and then sequence the relevant portion.

We are analyzing the role of strand breaks in meiosis in RAD^+ strains using a slightly different approach. There are regions of the yeast genome that have been shown to exhibit high levels of meiotic recombination. These regions are good candidates for preferred sites for the initiation of meiotic recombination and preliminary results suggest that site-specific single strand breaks can be

detected. We are currently examining the sites where the breaks are found. Consistent with our gradient results, double strand breaks are not found.

As noted above, there are mitotically identified repair gene functions that can be essential in meiosis. In the absence of some functions recombination is abolished and cells die from extensive aneuploidy or defective recombinational processes. The effects of damage induced in the DNA of RAD⁺ cells undergoing meiosis could be due to direct action on meiotic processes or alternatively the damage could decrease the availability of some repair gene products in normal recombinational processes. We will continue to characterize damage induced repair events that occur during meiosis. Since systems that enable extensive recombination are present during meiosis, damage which is repaired through recombinational mechanisms such as double-strand breaks might be repaired to a greater extent. While we have described excision repair during meiosis, the capability for repair will be examined more quantitatively. We have developed methods for isolating cells that exhibit enhanced meiotic synchrony which should enable us to characterize more precisely the capability for excision as well as other types of repair during meiosis. Using the procedure for probing unique regions of chromosomes we plan to examine repair in specific chromosomes and the extent to which replication can bypass unrepaired damage.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: DNA repair mechanisms are of fundamental importance in the process of mutagenesis and ultimately in carcinogenesis. Using yeast as a model lower eukaryote, we have been able to dissect and analyze at least two pathways of DNA repair at the molecular level in growing and meiotic cells. Since these pathways are involved in mutagenesis, this work will further our understanding of the basic mechanism of mutation. The repair mutants have also provided us with the first opportunity to identify specific changes in DNA that relate to meiotic recombination and relate them to genetic events. By characterizing the events that occur during meiosis and the role of the various repair genes, it is possible to understand how repair systems function to both protect cells and to enable meiotic recombination to occur. In the absence of some repair genes, aneuploidy and meiotic cell death occurs. These results imply that decreased levels of repair function possibly due to DNA damage can affect the overall DNA metabolic activities in meiocytes. The techniques which we are developing will be useful in identifying rare changes in DNA as well as specific changes in individual chromosomes.

PUBLICATIONS

Resnick, M.A., Chow, T., Nitiss, J., and Game, J.C.: Changes in the chromosomal DNA of yeast during meiosis in repair mutants and the possible role of a deoxyribonuclease. Cold Spring Harbor Symposium on Quantitative Biology 49: 639-649, 1984.

BIOMEDICAL SCIENCES DIVISION
LAWRENCE LIVERMORE NATIONAL LABORATORY
UNIVERSITY OF CALIFORNIA
LIVERMORE, CALIFORNIA 94550
(Y01-ES-1-0063)

TITLE: Mutagens from the cooking of foods

CONTRACTOR'S PROJECT DIRECTOR: Frederick T. Hatch, M.D.

PROJECT OFFICER (NIEHS): Errol Zeiger, Ph.D., Supervisory Microbiologist
Environmental Mutagenesis Group, CGTB

DATES AGREEMENT INITIATED: 1. September 22, 1978 (222-Y01-ES-80038)
2. April 1, 1981 (222-Y01-ES-10063)
3. April 1, 1984 (Y01-ES-10063)

CURRENT ANNUAL LEVEL: \$703,982

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this interagency agreement with the Department of Energy are to identify, isolate, and characterize the mutagens produced in foods, primarily beef products, cooked under approximately normal household conditions, and determine their mechanism(s) of formation, assess the spectrum of genetic toxicity caused by these mutagens using in vitro and in vivo short-term tests, and devise strategies to limit or prevent mutagen formation.

METHODS EMPLOYED: Hamburger is fried under normal cooking conditions, extracted, and the extracts tested for mutagenicity using the Salmonella plate test with S-9 preparations from rodents pretreated with various inducers. Extracts with the highest levels of mutagenicity are separated to isolate and identify the mutagenic components. Similar work is being done with other fried meats, fried eggs, and boiled beef extracts. Mutagenicity studies are also being performed in cultured Chinese hamster ovary (CHO) cells and in mice, in vivo. Metabolism studies are carried out using in vitro S-9 incubation. Standard chemistry procedures are used to isolate, purify, and identify the mutagenic components of these extracts.

MAJOR FINDINGS AND PROPOSED COURSE:

Fried, ground beef: The kinetics of mutagen formation during the frying of ground beef to a well-done, non-charred state have been determined. Mutagen formation is dependent on temperature, time of cooking, water content of the meat, and the heat-transfer properties of the surface on which it is cooked. The original procedure for extraction of mutagens has been modified and improved so that LLNL can now obtain a mutagen yield that is 100-fold greater than originally reported. At least 10 different mutagens are present in fried, ground beef. A number of these mutagens have been isolated and purified, only some of which have been previously identified. Some of the newly identified mutagens have been characterized structurally and methods for their chemical synthesis have been developed. They are being synthesized in quantities sufficient for in vitro and in vivo mutagenicity assays.

Boiled beef: Long-term boiling or simmering of beef in water results in the formation of mutagens. When the volume is held constant, mutagen formation is a function of time and pH. Addition of tryptophan, proline, and creatine to the boiling mixture increased mutagen formation; other amino acids and non-amino acid

amines did not increase activity. The mutagens formed, Trp-P-2 and IQ, are identical to mutagens isolated from other cooked food and pyrolyzed food preparations. Studies are underway to identify the components of the beef that are responsible for mutagen formation.

Two food-derived mutagens have been extensively characterized with respect to their mutagenicity in bacteria, cultured mammalian cells, and in mouse bone marrow. Trp-P-2 (3-amino-1-methyl-5H-pyrido [4,3-b] indole) was less mutagenic in *Salmonella* than IQ (2-amino-3-methylimidazo [4,5-f] quinoline), but Trp-P-2 was more mutagenic in mammalian cells (point mutations, chromosome aberrations and sister chromatid exchanges). Trp-P-2 and IQ both induced sister chromatid exchanges in mouse bone marrow; Trp-P-2, but not IQ, also induced chromosome aberrations. The in vitro metabolism of both mutagens is being studied and attempts are being made to identify and isolate the ultimate mutagens. Through the use of radiolabelled mutagens, the types and levels of DNA adducts formed have been measured, and these data will be used in an attempt to explain the differences between bacterial and mammalian cell responses.

Ten newly identified pure mutagens are being synthesized to confirm their structures, after which they will be characterized as to their genetic activity in bacteria, cultured mammalian cells, and rodents. Radiolabelled mutagens are being synthesized and pharmacokinetic studies will be performed in rodents.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is well accepted that mutagenic substances are normal components of the environment. In order to place human exposure levels to food mutagens in their proper perspective, it is important to characterize the levels and types of exposure and the biological activities of the mutagens.

PUBLICATIONS

Felton, J.S., Hatch, F.T., Knize, M.G., and Bjeldanes, L.F.: Mutagens in Cooked Beef: Characterization and Genotoxic Effects. In Campbell, T.C. and Roe, D. (Eds.): Diet and Cancer: From Basic Research to Policy Implications. New York, Alan R. Liss, 1983, pp. 177-194.

Bjeldanes, L.F., Felton, J.S., and Hatch, F.T.: Mutagens in Cooked Foods: Chemical Aspects. In Friedman, M. (Ed.): Nutritional and Metabolic Aspects of Food Safety, Advances in Experimental Medicine and Biology. New York, Plenum Press, 1984, pp. 545-554.

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Hatch, F.T., Felton, J.S., Stuermer, D.H., and Bjeldanes, L.F.: Identification of Mutagens from the Cooking of Food. In de Serres, F.J. and Hollaender, A. (Eds.): Chemical Mutagens: Principles and Methods for Their Detection, Vol. 9, Chap. 6. New York, Plenum Press, 1984, pp. 111-164.

Minkler, J.L., and Carrano, A.V.: In vivo cytogenetic effects of the cooked-food-related mutagens Trp-P-2 and IQ in mouse bone marrow. Mutat. Res. Lett. 140: 49-53, 1984.

Taylor, R.T., Fultz, E., and Shore, V.: Mutagen formation in a model beef boiling system. I. Basic characteristics of the system. J. Environ. Sci. Health A19: 819-845, 1984.

Taylor, R.T., Shore, V., and Fultz, E.: Mutagen formation in a model beef boiling system. II. Effects of proteolysis and comparison of soluble fractions from several protein sources. J. Environ. Sci. Health A19: 819-845, 1984.

Felton, J.S., Bjeldanes, L.F., Rapoport, H., and Hatch, F.T. In Hilado, C.J. (Ed.): Proc. of Fifth California Conf. on Product Toxicity. Sunnyvale, CA, Publ. Product Safety Corp., 1984, Vol. 5, pp. 11-16.

Taylor, R.T., Fultz, E., and Knize, M. Mutagen formation in a model beef boiling system. III. Purification and identification of three heterocyclic amine mutagens-carcinogens. J. Environ. Sci. and Health A20: 135-148, 1985.

Brookman, K.W., Salazar, E.P., and Thompson, L.H. Comparative mutagenic efficiencies of the DNA adducts from cooked-food-related mutagens Trp-P-2 and IQ in CHO cells. Mutation Res. 149: 249-255, 1985.

Felton, J.S., and Knize, M.G. Letter to the Editor. Chemtech. 15: 2, 1985.

Waterhouse, A.L., and Rapoport, H. Synthesis and tritium labeling of the food mutagens IQ and methyl IQ. J. Labeled Cpds. and Radiopharm., in press.

Knize, M.G., Andresen, B.D., Healy, S.K., Shen, N.H., Lewis, P.R., Bjeldanes, L.F., Hatch, F.T., and Felton, J.S. Effect of temperature, patty thickness and fat content on the production of mutagens in fried ground beef. Food and Chemical Toxicology, in press.

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37831
(Y01-ES-10067)

TITLE: Potential Hazard from Chemically Induced Transmitted Gene Mutations
Using the Specific Locus Method in Mice

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Liane B. Russell

PROJECT OFFICER (NIEHS): Michael D. Shelby, Ph.D., Head
Mammalian Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: April 15, 1981

CURRENT ANNUAL LEVEL: \$491,023

PROJECT DESCRIPTION

OBJECTIVES: The first objective of this project is to investigate chemically-induced mutation processes in mice using the germ-cell mutagen N-ethyl-N-nitrosourea (ENU). This compound is sufficiently mutagenic to permit the study of cell stage sensitivities, dose response curves, and effects on both male and female germ cells. The second objective is to test chemicals of environmental significance for germ-cell mutagenicity.

METHODS EMPLOYED: Induced mutant frequencies are determined by administering ENU or the test chemical to one parent, usually the male, that is homozygous wild-type for seven morphological markers (primarily coat color markers). The treated parent is mated to the untreated parent which is homozygous recessive at the same seven loci. Mutant offspring are detected at 3-4 weeks of age as those exhibiting a visible recessive trait among the normal offspring that appear as wild-type.

MAJOR FINDINGS AND PROPOSED COURSE: Negative results for 1,2-dibromo-3-chloropropane (DBCP) in the specific locus and dominant lethal tests led to additional studies because DBCP is a carcinogen in mice and a dominant lethal inducer in rats. Both mouse strains 101 and 101 x C3H hybrids were tested and shown to be Ah-responsive phenotypes. DBCP induction of unscheduled DNA synthesis in early spermatids of seven mouse strains was tested. In all cases, negative or marginal responses were observed.

Final analysis of urethane data shows that it does not induce specific locus mutations although there is published evidence that urethane reaches the male germ cells. 6-Mercaptopurine results are clearly negative for post-stem cell stages and not statistically significantly different from controls for treated stem cells. Ethylene dibromide (EDB) was shown to bind to germ cell DNA, but specific locus studies with single and multiple injections of EDB yielded clearly negative results.

Dose fractionation studies with ethylnitrosourea (ENU) have been extended to 20 fractions of 5 mg/kg with a resulting induced mutation rate that is only 62% of the rate obtained with 10 fractions of 10 mg/kg, again indicating the possibility

of efficient repair at low dose rates. In ENU studies of dosed females, > 4000 progeny conceived in the first six weeks after exposure and > 7000 progeny conceived in later weeks have yielded no evidence of mutation induction. Dosimetry studies in males treated with very low doses of tritiated ENU have provided evidence that even at a dose of 0.8 mg/kg, ENU binding to testicular DNA appears to be linear.

Additional chemicals will be tested for germ cell mutagenicity and ENU studies will be continued in the areas of age effects, male post-meiotic germ cell effects, and molecular dosimetry.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is known that humans are exposed to mutagenic chemicals and mutations are the basis for a significant portion of human disease. This project is designed to contribute to the protection of human health through further understanding of the process of induced mutations in mammalian germ cells, assessing the mutagenicity of selected, environmentally significant chemicals, and contributing data for use in genetic risk estimation efforts.

ENVIRONMENTAL MUTAGEN INFORMATION CENTER
(Department of Energy)
Oak Ridge, TN 37830
(Y01-ES-10072)

TITLE: Core and Non-core Activities of the Environmental Mutagen Information Center

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Mr. John S. Wassom

PROJECT OFFICER (NIEHS): Stanley Stasiewicz
Cellular and Genetic Toxicology Branch

DATE CONTRACT INITIATED: July 1, 1983

CURRENT ANNUAL LEVEL: \$439,000

PROJECT DESCRIPTION

OBJECTIVES: The Environmental Mutagen Information Center (EMIC) collects, organizes, and disseminates, published information on chemicals tested for various endpoints of genetic toxicity. The information is entered into a computerized data file that can be readily used by scientists interested in the discipline of genetic toxicology.

METHODS: The EMIC scans key journals as well as secondary literature sources for reports in the open literature and obtains copies of those reports for entry into their data file. Papers selected for entry into the file contain information related to the testing of chemicals (or other environmental agents excluding papers dealing solely with ultraviolet and ionizing radiation) for mutagenicity. Papers are also selected that contain information on peripheral subjects that may be useful in understanding the known or suspected mutagenic activity of environmental agents. The EMIC maintains and constantly updates a computerized data file which contains for each published record entered, bibliographic information, assay system, and key words defining agents tested and organisms studied. This data file is available on-line from TOXLINE (National Library of Medicine) and from RECON (Oak Ridge National Laboratory).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project aids federal agencies and others to rapidly and thoroughly access the world's literature in the area of genetic toxicology. The EMIC file contains (as of May 1985) over 55,393 literature citations on more than 20,000 agents. This system is used to keep abreast of short-term test development and validation and to develop improved test methods for assessing the mutagenic and carcinogenic potential of environmental agents.

UNIVERSITY OF CALIFORNIA
Berkeley, California 94703
(N01-ES-1-5004)

TECHNISCHE HOCHSCHULE
Darmstadt, Germany
(N01-ES-1-5005)

TITLE: Development of a Yeast Aneuploidy Test System

CONTRACTOR'S PROJECT DIRECTOR: Seymour Fogel, Ph.D. (N01-ES-1-5004)
Friedrich Zimmermann, Ph.D. (N01-ES-1-5005)

PROJECT OFFICER (NIEHS): Michael A. Resnick, Ph.D., Research Geneticist
Environmental Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: July 1, 1981

CURRENT ANNUAL LEVEL: N01-ES-1-5004 = \$115,000
N01-ES-1-5004 = \$20,000

OBJECTIVES: The purpose of these contracts is to develop strains and procedures with the yeast Saccharomyces cerevisiae which will enable the rapid screening of agents that induce aneuploidy during meiotic development and mitotic growth. These strains will enable a comparison of the genetic effects of agents in terms of the induction of recombination and mutation as well as aneuploidy. The yeast aneuploidy test system will be evaluated for use as an integral component in a battery of tests used to detect genetically active agents.

METHODS EMPLOYED: In the development of the meiotic portion of the aneuploidy test system, strains have been devised for following chromosome gain among the haploid products of meiosis. Aneuploidy is detected by increased gene dosage at the ARG4 locus, which would correspond to chromosome gain; the methods involved can be adapted to aneuploidy in mitotic cells. Aneuploidy in mitotically growing cells is being detected as chromosome loss as evidenced by the appearance of a drug-resistant recessive marker. Using the strains that have been developed, the contractors are determining the most effective protocols for a rapid screen of chemicals. The methods for testing will be based on results obtained with a series of positive controls and coded chemicals supplied by NIEHS. After a protocol has been defined, it will be validated between two laboratories by screening a number of coded chemicals.

MAJOR FINDINGS AND PROPOSED COURSE: The strain used for detecting mitotic aneuploidy is a refinement of a previously published strain in that it enables clearer discrimination against false positives. Several compounds previously reported as causing aneuploidy have not been found to be positive in this refined system. In some cases the reported cases of aneuploidy may have been due to other genetic events mimicking an aneuploidy response. Procedures of analysis have been developed to enable discrimination between aneuploidy and other events. Several chemicals have been identified as causing aneuploidy. A major category, consisting largely of aprotic polar solvents such as acetone, involves an intervening cold shock period during treatment to elicit a high level of response, although aneuploidy is also observed at the normal growth temperature. Based on genetic evidence, these agents act primarily on non-DNA targets. The mode of action appears to be related to tubulin aggregation into microtubules, with some chemicals stabilizing and other destabilizing the microtubules. Using several structurally related chemicals, it has been possible to determine structure versus aneuploidy induction relationships. A validation phase has begun with the mitotic chromosome loss system using coded chemicals.

The strains for detecting meiotic aneuploidy are being developed with several genetic markers which enable a clear discrimination between haploid cells that are aneuploid or false positive. Conditions are also being investigated to allow for clearer discrimination of spores containing extra chromosomes. The system is very sensitive so that aneuploidy can be detected at doses which do not lead to appreciable losses in sporulation; agents which induce mitotic aneuploidy have also been shown to induce meiotic aneuploidy.

The term of the current contracts ends June 30, 1985, and they are being re-competed. The new contracts will begin in the summer of 1985 and will be devoted to continued development of the systems, protocol development, validation, and testing of chemicals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Aneuploidy contributes significantly to the genetically based disease burden in human populations with approximately 0.4% of live births exhibiting abnormal chromosome numbers. A large fraction of spontaneous abortion in humans and certain serious genetic diseases (e.g., Down's syndrome) are caused by aneuploidy. Aneuploidy has also been implicated in some steps in tumor production. A few chemicals are known which only induce aneuploidy in genetic test systems; however, there is, in fact, no reliable, well-developed rapid screen to detect such agents on a large scale. A yeast test system that detects mitotic and meiotic aneuploidy will enable the future rapid screening of chemicals and agents 1) that induce gross chromosomal changes which would not be identified as mutagenic in microbial test systems, and/or 2) that cause changes in chromosomal number in addition to being mutagenic or recombinogenic.

PUBLICATIONS

Zimmermann, F.D., Mayer, V.W., and Scheel, I.: Induction of aneuploidy by oncodazole (Nocodazole), an antitubulin agent, and acetone. Mutat. Res. 141: 15-18, 1984.

Groschel-Stewart, U., Mayer, V.W., Taylor-Mayer, R.E., and Zimmermann, F.K.: Aprotic polar solvents inducing chromosome malsegregation in yeast interfere with the assembly of porcine brain tubulin in vitro. Mutat. Res. 149: 333-338, 1985.

Zimmermann, F.D., Mayer, V.W., Scheel, I., and Resnick, M.A.: Acetone, methyl ethyl ketone, ethyl acetate, acetonitrile and other polar aprotic solvents are strong inducers of aneuploidy. Mutat. Res. 149: 339-345, 1985.

Zimmermann, F.K., Groschel-Stewart, U., Scheel, I., and Resnick, M.A.: Genetic change may be caused by interference with protein-protein interactions. Mutation Res., in press.

ENVIRONMENTAL HEALTH RESEARCH AND TESTING Lexington, Kentucky 40503 (N01-ES-1-5789)	BIOASSAY SYSTEMS CORP. Woburn, Massachusetts 01801 (N01-ES-3-5029)	LITTON BIONETICS, INC. Kensington, Maryland 20795 (N01-ES-3-5030)
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TITLE: In Vitro Cytogenetic Testing

CONTRACTOR'S PROJECT DIRECTOR: Dushyant Gulati, Ph.D. (N01-ES-1-5789)
Ken Loveday, Ph.D. (N01-ES-3-5029)
James Ivett, Ph.D. (N01-ES-3-5030)

PROJECT OFFICERS (NIEHS): Errol Zeiger, Ph.D., Supervisory Microbiologist
Michael A. Resnick, Ph.D., Research Geneticist
Michael D. Snelby, Ph.D., Geneticist
Environmental Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: N01-ES-1-5789: September 30, 1981
N01-ES-3-5029 and N01-ES-3-5030: September 29, 1983

CURRENT ANNUAL LEVEL: N01-ES-1-5789 = \$465,734
N01-ES-3-5029 = \$247,971
N01-ES-3-5030 = \$297,810

PROJECT DESCRIPTION

The purpose of these contracts is to test approximately 375 chemicals over a period of 4 years for their ability to induce chromosome aberrations and sister chromatid exchanges in cultured Chinese hamster ovary (CHO) cells. Protocol development and validation of the CHO cytogenetics assay was conducted earlier under contracts with Columbia University and Litton Bionetics, Inc.

METHODS EMPLOYED: Chinese hamster ovary cells in culture are used to test for the induction of chromosome aberrations and sister chromatid exchange, both with and without S-9 preparations from Aroclor 1254-induced Sprague-Dawley rats. Results obtained from testing the coded substances are entered on standardized data forms and transferred to a computerized database management system. The results are analyzed statistically to determine responses.

MAJOR FINDINGS AND PROPOSED COURSE: Since the inception of the CHO cytogenetics contracts in 1979, a total of 357 samples (including 319 unique chemicals) have been tested. Protocols have been developed and validated which allow for high levels of accuracy and reproducibility in the testing of chemicals for in vitro cytogenetic effects. Through an in-depth analysis of the methods being used in the various laboratories, uniform procedures have been established for testing, scoring, and data presentation. Data entry and statistical evaluation systems have been established within NIEHS for analysis of results.

The accumulated data have enabled the development of rigorous and statistically sound criteria for evaluation of chemical responses, based in part on the biological responsiveness of the system. To evaluate the sensitivity and reproducibility of the system for detecting small increases in SCEs, low concentrations of positive control chemicals were routinely included as weak positive controls during tests of coded compounds. Based on nearly 100 tests, it was determined that the detection of small increases over background was highly reproducible. This

approach has allowed the direct evaluation of the resolving power of the system. The inclusion of a weak positive control is now routinely included as part of the protocol to evaluate the responsiveness of the system during a test of a coded compound.

Nearly all chemicals which are positive in chromosome aberration tests are also positive in the sister chromatid exchange tests, but the converse is not true. Also, several chemicals which have been shown to be negative in the Salmonella assay are positive in the cytogenetic assays, and vice versa. The cytogenetics results with 77 compounds which have also been tested for carcinogenesis in the NTP whole animal program are being compared with those from other systems being used in CGTB. The purpose of this will be to evaluate parameters which may be useful in predicting the carcinogenicity of chemicals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The Salmonella test system currently in use is designed to detect substances which induce point mutations. It is not capable of detecting substances that produce only chromosome mutations. The Chinese hamster ovary cell system will allow detection of chemicals which do not produce point mutations in Salmonella but are capable of producing chromosome aberrations in cultured mammalian cells. Sister chromatid exchange is being used as an additional indicator for substances that are capable of damaging mammalian chromosomes. Results from these tests in conjunction with results from other genetic toxicity tests will be used to assist in decisions regarding chemicals to be tested in subchronic and chronic toxicological tests, as well as in vivo cytogenetics tests.

PUBLICATIONS

Gulati, D.K., Sabharwal, P.S., and Shelby, M.D.: Testing of 10 IPCS Chemicals for Induction of Cytogenetic Damage in CHO Cells. In Ashby, J. et al. (Eds.): Collaborative Study of Short-Term Tests for Carcinogens, Amsterdam, Elsevier/North Holland, in press.

Galloway, S.M., Bloom, A.D., Resnick, M.A., Margolin, B.H., Nakamura, F., Archer, P., and Zeiger, E.: Development of a standard protocol for in vitro cytogenetic testing with CHO cells: Comparison of results for 22 compounds in two laboratories. Environ. Mutagen. 7: 1-52, 1984.

ARGONNE NATIONAL LABORATORY
Argonne, Illinois 60439
(Y01-ES-20102)

ARTHUR D. LITTLE, INC.
Cambridge, MA 02140
(N01-ES-15794)

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20016
(N01-ES-15758)

TITLE: Task I - Mammalian Cell Transformation Using Syrian Golden Hamster Embryo Cell Culture Using the Colony Transformation Endpoint

CONTRACTORS' PRINCIPAL INVESTIGATORS: Drs. E. Huberman and C. Jones (N01-ES-20102)
Drs. A. Tu and A. Sivak (N01-ES-15794)
Dr. R. Lubet (N01-ES-15758)

PROJECT OFFICER (NIEHS): Raymond W. Tennant, Ph.D., Chief, Cellular and Genetic Toxicology Branch

DATE CONTRACT INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: Y01-ES-20102 = \$353,304
N01-ES-15794 = \$312,989
N01-ES-15758 = \$315,511

PROJECT DESCRIPTION

OBJECTIVES: This project is a three-laboratory evaluation, using coded chemicals, of the Syrian hamster embryo neoplastic transformation assay for detection of potential chemical carcinogens. Initial objectives involve the development of a standardized test protocol, identification of the sources of intra- and interlaboratory variability and establishment of interlaboratory reproducibility of the test system. Results of previous contract-supported studies and published results have shown that the SHE transformation assay detects chemical carcinogens. This project is one part of an effort to systematically evaluate and compare three assays for neoplastic transformation to determine which system may be most useful in identifying or distinguishing chemical carcinogens.

METHODS EMPLOYED: Syrian hamster embryo cells are collected, frozen, characterized for their response to known carcinogens, and then exposed to concentrations of the test chemical, based upon previous tests for toxicity. After 7-10 days, treated cultures are examined for foci of transformed cells.

MAJOR FINDINGS AND PROPOSED COURSE: Major goals of this project have included: 1) the standardization of a test protocol; 2) identification of key test reagents and materials; 3) selection of optimal lots of reagents and materials; following preliminary testing; 4) acquisition of sufficient quantities of critical reagents; and 5) tests of representative rodent carcinogenesis assay positive and negative chemicals for toxicity and transformation to establish interlaboratory reproducibility of the methods. Each contract laboratory also had the responsibility of focusing on key components of the test system (e.g. identification of suitable frozen cell pools; identification of optimal serum and medium stocks). Progress has been made in all these areas, although some technical aspects require further evaluation. A manuscript detailing the results from the comparative interlaboratory evaluation of model and selected coded chemicals has been accepted for publication. Working criteria for acceptability and evaluation of the assay have been defined, though these criteria

may be modified as more data are evaluated. Because of some difficulties encountered with various technical aspects of this assay, work has progressed at a slower rate than expected. Therefore, the contracts were extended for one year. During this time, additional coded chemicals are being tested to confirm the interlaboratory reproducibility and work on resolving technical differences will continue. This data will be included in a second joint manuscript.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A variety of independent studies have shown a high correlation between the ability of chemicals which are known to induce tumors in vivo to induce neoplastic transformation in certain cultured mammalian cells. Such in vitro systems offer significant advantages in time and cost over animal bioassays for carcinogens. In addition, they often provide information for mechanistic inferences on the toxicity of chemicals. It is important that the use of such test systems include the application of standardized protocols which provide a high degree of interlaboratory reproducibility and an understanding of the biological limitations of the test system.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20016
(N01-ES-15795)

NORTHROP SERVICES, INC.
Research Triangle Park, NC 27709
(N01-ES-15796)

TITLE: Task II - Mammalian Cell Transformation using Syrian Hamster Embryo (SHE)
Cells Infected with Simian Adenovirus (SA7)

CONTRACTORS' PRINCIPAL INVESTIGATORS: Dr. Ronald Lubet (N01-ES-15795)
Dr. George Hatch (N01-ES-15796)

PROJECT OFFICER (NIEHS): Raymond W. Tennant, Ph.D., Chief, Cellular and Genetic
Toxicology Branch, and Judson Spalding, Ph.D. (Co-project
Officer)

DATE CONTRACT INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: N01-ES-15795 = \$356,325
N01-ES-15796 = \$487,969

PROJECT DESCRIPTION

OBJECTIVES: This project is a dual laboratory evaluation using coded chemicals, of the SA7/SHE transformation assay system for detecting potential chemical carcinogens. Initial objectives involve: 1) the development of a standardized test protocol; 2) the identification of the sources of intra- and interlaboratory variability; and 3) the establishment of the interlaboratory reproducibility of the test system. Published results on the SA7/SHE transformation enhancement assay indicate that the system detects chemicals of known carcinogenic potential, and may be particularly useful in the identification of potential carcinogens from some specific chemical classes which are not easily detected in other assays for genetic toxicity. This project is one part of an effort to systematically evaluate and compare three assays for neoplastic transformation to determine which system may be most useful in identifying chemical carcinogens.

METHODS EMPLOYED: Primary cultures of SHE cells are prepared from pooled 13 day gestation embryos; transforming virus is obtained from standardized frozen stocks of SA7 with defined PFU/FFU ratio. Cells are infected with virus prior to or after treatment with doses of test chemical, that have been selected on the basis of previously determined toxicity. Cultures are scored for toxicity and transformed foci after 7-9 days of cultivation and the transformation frequency and enhancement ratio for each chemical is determined.

MAJOR FINDINGS AND PROPOSED COURSE: Major goals of this project have included: 1) standardization of the test protocol; 2) identification of key test reagents and materials; 3) selection of optimal lots of reagents and materials; 4) acquisition of sufficient quantities of critical reagents; and 5) tests of representative rodent carcinogenesis assay positive and negative chemicals for toxicity and transformation to establish the interlaboratory reproducibility of the methods. These goals have been met and technical aspects are being clarified. The protocol has been modified to optimize the assay's response to chemical treatment and working criteria for acceptability and evaluation of the

assay have been adopted. Other goals of the project were: preparation of joint publications detailing the results of work on model and coded chemicals; having the two laboratories perform toxicity and transformation assays on a group of coded compounds; comparison of the results to determine the degree of interlaboratory reproducibility; resolution of any technical differences and establishment of standard criteria for acceptability and evaluation; and establishment of a standard protocol. Progress has been seen in all these areas. Though there has been progress, there have been some difficulties encountered which caused the project to proceed at a much slower rate than expected. Therefore, the contracts were extended for one year. During this time, work will be directed towards reducing the intra- and interlaboratory variability and testing more selected coded chemicals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A variety of independent studies have shown a high correlation between the ability of chemicals which are known to induce tumors *in vivo* to induce oncogenic transformation in certain cultured mammalian cells. Such *in vitro* systems offer significant advantages in time and cost over animal bioassays for carcinogens. In addition, they often provide information for mechanistic inferences on the toxicity of chemicals. It is important that the use of such test systems include the application of standardized protocols which provide a high degree of interlaboratory reproducibility and an understanding of the biological limitations of the test system.

BIOTECH RESEARCH LABS
Rockville, Maryland 20014
(N01-ES-15807)

LITTON BIONETICS
Kensington, MD 20895
(N01-ES-15797)

NORTHROP SERVICES, INC.
Research Triangle Park, NC 27709
(N01-ES-15798)

TITLE: Task III - Mammalian Cell Transformation Retrovirus Infected Rat Cells

CONTRACTORS' PRINCIPAL INVESTIGATORS: Drs. A. Steuer and R. Ting (N01-ES-15807)
Drs. J. Poiley and R. Raineri (N01-ES-15797)
Dr. W. Suk (N01-ES-15798)

PROJECT OFFICER (NIEHS): Raymond W. Tennant, Ph.D., Chief, Cellular and Genetic
Toxicology Branch

DATE CONTRACT INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: N01-ES-15807 = \$152,291
N01-ES-15797 = \$454,392
N01-ES-15798 = \$472,711

PROJECT DESCRIPTION

OBJECTIVES: This project is a three-laboratory evaluation, using coded chemicals, of the rat cells infected with the Rauscher leukemia virus (2FR,50) neoplastic transformation assay for detection of potential chemical carcinogens. Initial objectives involve the development of a standardized test protocol, identification of the sources of intra- and interlaboratory variability and establishment of interlaboratory reproducibility of the test system. Results of previous contract-supported studies and published results have shown that this transformation assay detects chemical carcinogens. This project is one part of an effort to systematically evaluate and compare three assays for neoplastic transformation to determine which system may be most useful in identifying and distinguishing chemical carcinogens.

METHODS EMPLOYED: The infected (2FR,50) and uninfected (2FRN) cell lines obtained from American Type Culture Collection were cultivated from passage 7. The cells are first exposed to chemicals to determine the toxicity and subsequently appropriate doses are applied and the cells are tested for transformation by a modified aggregation (survival) assay which detects the preferential ability of transformed cells to survive in an anchorage-independent state.

MAJOR FINDINGS AND PROPOSED COURSE: Major goals of this project were: 1) the standardization of a test protocol; 2) identification of key test reagents and materials; 3) selection of optimal lots and acquisition of sufficient quantities of reagents and materials; and 4) tests of representative rodent carcinogenesis assay positive and negative chemicals for toxicity and transformation to establish interlaboratory reproducibility of the methods. These goals have been met, although some technical aspects require further evaluation. The protocol has been modified to optimize the assay's response to chemical treatment and working criteria for acceptability and evaluation of the assay have been adopted, though they may be modified as more data are evaluated. Other goals achieved were: the preparation and publication of a joint manuscript detailing the results from the comparative evaluation of model and coded chemicals and

having the three labs achieve a level of interlaboratory reproducibility in the performance of the assay on a group of selected coded chemicals. These results are being evaluated and will be compiled for a second joint manuscript. Because of difficulties encountered with various aspects of the assay, work had progressed at a rate slower than expected, therefore the contracts were extended for one year. During that time, basic research into the mechanism of the assay and ways to enhance sensitivity as well as alternative endpoints have been pursued.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A variety of independent studies have shown a high correlation between the ability of chemicals which are known to induce tumors in vivo to induce oncogenic transformation in certain cultured mammalian cells. Such in vitro systems offer significant advantages in time and cost over animal bioassays for carcinogens. In addition, they often provide information for mechanistic inferences on the toxicity of chemicals. It is important that the use of such test systems include the application of standardized protocols which provide a high degree of interlaboratory reproducibility and an understanding of the biological limitations of the test system.

ENVIRONMENTAL PROTECTION AGENCY
Research Triangle Park, NC 27709
(Y01-ES-20079)

TITLE: Coordinated Testing Project (CGT Rapid Test Response, Component II)

WORK PERFORMED AT THE FOLLOWING CONTRACT LABORATORIES:

SRI International	Litton Bionetics, Inc.
(Dr. Jon Mirsalis, PI)	(Dr. John Rundell, Co-PI, and Dr. Edward Matthews, Co-PI)

PROJECT OFFICER (NIEHS): Judson W. Spalding, Ph.D.
Cellular and Genetic Toxicology Branch

PROJECT OFFICER (EPA): Stephen Nesnow, Ph.D., Experimental Toxicology
Division, HERL

DATE CONTRACT INITIATED: November 29, 1982.

CURRENT ANNUAL LEVEL: \$220,000

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project was to test up to 20 chemicals in the in vivo-in vitro (host activated) rodent hepatocyte DNA repair and hepatotoxicity assay, which is one component of a complimentary group of short-term tests which were selected to provide multiple test systems and endpoints. The in vivo-in vitro hepatocyte DNA damage/repair assay and hepatotoxicity studies were performed in both rat (F344) and mouse (B6C3F₁) hepatocytes. These additional objectives were included in order to investigate possible differences in species sensitivity to chemical genetic toxicity and to distinguish those chemicals that were hepatotoxic rather than directly genotoxic. The results from these tests will contribute substantially to a data base which will permit an evaluation of the genetic toxicity potential of the chemicals tested.

The contract at Litton Bionetics, Inc. for the performance of the in vitro transformation of Balb/c 3T3 cells assay was terminated July 31, 1984. During the latter part of this contract, the assay protocol was modified to include a metabolic activation component.

METHODS EMPLOYED: The chemicals selected were usually coded and, when possible, they were taken from the same chemical lot that has been prepared for the bioassay. The chemicals were tested according to established protocols for the in vivo-in vitro (host activated) rat hepatocyte DNA damage/repair assay which was also adapted for the use of mouse hepatocytes. Additional protocols were established for hepatotoxicity studies in primary hepatocytes from pretreated rats and mice.

In order to increase the sensitivity of the assay, the protocol was modified to include a metabolic activation component which was provided by co-cultivation of isolated rat hepatocytes with the target Balb/c 3T3 cells.

MAJOR FINDINGS AND PROPOSED COURSE: The contract at Litton Bionetics, Inc. for one of the two test components, the in vitro transformation of Balb/c 3T3 cell assay was terminated July 13, 1984. Twenty-nine chemicals were tested in the last year of this contract. Over one-half of these chemicals were tested in both the standard assay and in a modified assay that included co-cultivation with isolated rat hepatocytes as a metabolic activation component. Twenty-four of the 30 chemicals tested in the previous year were also retested in the modified protocol. The inclusion of the metabolic activation component significantly increased the sensitivity of the assay. These test results have been compiled and evaluated, and were recently submitted in a manuscript for publication. It is the intent to continue testing chemicals in this in vitro transformation assay. A recent RFP issued by CGTB/NTP includes a provision for the further testing of chemicals in this assay.

The contract for the second test component, the in vivo-in vitro rodent hepatocyte DNA repair and hepatotoxicity assay was continued for another year, but will terminate July 31, 1985. The chemicals selected for testing in this assay were primarily those chemicals that had been identified as hepatocarcinogens in the NTP rodent carcinogenesis assay. Over one-half of these hepatocarcinogens failed to induce in vivo-in vitro UDS in the same sex/species in which the incidence of liver tumors was increased in the two-year rodent carcinogenesis study. In addition, these chemicals exhibited weak or inconsistent genetic toxicity in other genetic toxicity tests. However, these chemicals were able to induce a proliferative (S-phase) response which is an indicator of hepatotoxicity. This evidence suggests that those hepatocarcinogens which exhibit little or no genetic toxicity are promoting the development of hepatocellular tumors that may be spontaneously or heritably initiated. The in vivo-in vitro rodent hepatocyte DNA repair and hepatotoxicity assay has the potential for distinguishing between hepatocarcinogens that are genotoxic and those which exhibit promoter activity. The results from these tests will be evaluated and compiled for publication at the termination of the contract. It is the intent to continue evaluating NTP hepatocarcinogens and other chemicals in this assay system. An RFP for this purpose will be initiated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The Cellular and Genetic Toxicology Branch has the responsibility for providing short-term test support to the carcinogenicity bioassay program. A variety of short-term tests have been proposed to predict the potential carcinogenicity of chemicals. Up to now, current data from short-term tests have not been sufficient to predict the potential carcinogenicity of those chemicals submitted for test in the two-year rodent bioassay. The short-term assays described in this project are included in two of the four broad classes of in vitro short-term tests selected by CGTB to characterize the genotoxicity potential of chemicals. The information obtained from this "Rapid in vitro Test" capability may be utilized by experimental design groups and in the ranking process for establishing the priority of chemicals to be entered into the long-term carcinogenicity assays.

SCIENCE APPLICATIONS, INC.
(Department of Energy)
Oak Ridge, TN 37830
(Y01-ES-20080)

TITLE: Literature Review and Analysis on Environmental Mutagenesis

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Jack Tobler

PROJECT OFFICER (NIEHS): Stanley Stasiewicz
Cellular and Genetic Toxicology Branch

DATE CONTRACT INITIATED: April 1, 1984

CURRENT ANNUAL LEVEL: \$179,897

PROJECT DESCRIPTION

OBJECTIVES: To identify the biologically relevant metabolites, structurally related chemicals, and optical isomers for chemicals in the NTP rodent chronic toxicity bioassay. A literature search will identify the published information on the mutagenic, clastogenic, and other genetic toxicity properties of the parent compound and the identified related compounds. Significant literature (defined as that literature which meets the criteria used by the EPA Gene-Tox Panel) will be used in writing a report outlining the assay systems used and the results obtained with each system. The reports will also point out discrepancies in the results between similar tests, and, to the extent possible offer an explanation for the differences. These reports will provide a summary of the genetic toxicology information from the published literature for the NTP chemical managers and will be incorporated into reports on the two-year rodent bioassay.

METHODS: Identify the biologically relevant metabolites, structurally related chemicals, and optical isomers via commercial data bases and RECON/EMIC.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These reports provide an up to date summary and evaluation of the genetic toxicology information that is available in the published literature for bioassay chemicals, biologically relevant metabolites, structurally related chemicals, and optical isomers.

OAK RIDGE ASSOCIATED UNIVERSITIES
Oak Ridge, Tennessee 37830
(Y01-ES-20084)

TITLE: Genetic Toxicity Data Evaluation and Analysis Support

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. David M. DeMarini

PROJECT OFFICER (NIEHS): Michael D. Shelby, Ph.D., Head
Mammalian Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: November 1, 1982

CURRENT ANNUAL LEVEL: \$19,500

PROJECT DESCRIPTION

OBJECTIVES: This project is designed to collect, review, analyze, and summarize the genetic toxicology data on NTP priority chemicals. Data from the literature and from NTP testing projects are to be evaluated and included in the NTP Toxicology and Carcinogenesis Technical Reports.

METHODS EMPLOYED: Literature searches are performed using the bibliographic files of the Environmental Mutagen Information Center. Relevant papers are found, read, analyzed, and summarized. The CGTB's genetic toxicology data are summarized into tables and analyzed along with the results from the literature. Genetic toxicity profiles are summarized and discussed in relation to the carcinogenicity of the chemicals.

MAJOR FINDINGS AND PROPOSED COURSE: The genetic toxicology sections of 11 NTP Technical Report final drafts were completed. These reports include genetic toxicology sections in both the introductions and discussions and include data from both published literature and the CGTB testing program. The chemicals are dimethyl morpholinophosphoramidate (DMMPA), chlorinated trisodium phosphate, isophorone, benzyl acetate, 3-chloro-2-methylpropene, HC Red 3, C.I. Disperse Blue 1, Basic Red 9, o-phenylphenol, methylene chloride, and 4-vinylcyclohexene. Early versions of similar reports were prepared on trichloroethylene, tetrachloroethylene and 1,2-epoxyhexadecane.

Reviews of genetic toxicology data were prepared for the NTP Toxicology Design Committee on methylphenidate HCl, molybdenum trioxide, t-butanol, cobalt sulphate, m-nitrobenzoylchloride, p-nitrobenzoylchloride, barium chloride, methylene chloride, 1,1,1-trichloroethane, tungsten carbide, vinylidene fluoride, isobutyl nitrite, bromoform, propantheline bromide, 1,2-dichloropropane, 1-chloro-2-propanol, and 2-chloro-1-propanol. Much of the scientific activity of this project will soon be taken on by staff members of the CGTB, leaving the majority of effort on the contract to literature acquisition, data extraction and compilation, liaison with chemical managers, and scheduling of report and meeting preparations.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A substantial body of evidence indicates that alteration of the genetic apparatus of a cell is a necessary step in carcinogenesis. This idea is considered under the Somatic Mutation Theory of Cancer, which is currently a major working hypothesis regarding the way in which chemicals cause cancer. Because most chemical carcinogens are also mutagens, it is instructive to compare the mutagenicity and carcinogenicity of chemicals as an approach to elucidating the mechanisms of chemical carcinogenesis.

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37830
(Y01-ES-20085)

TITLE: Heritable Translocation Tests in Mice

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. W. M. Generoso

PROJECT OFFICER (NIEHS): Michael D. Shelby, Ph.D., Head
Mammalian Mutagenesis Group, CGTB

DATE INITIATED: June 1, 1982

CURRENT ANNUAL LEVEL: \$240,371

PROJECT DESCRIPTION

OBJECTIVES: This project is being conducted to obtain test data on the capacity of chemicals to induce heritable translocations in mammalian germ cells. Test chemicals will be selected primarily from those being tested in mouse specific locus assays.

METHODS EMPLOYED: Routes of administration are determined individually for each chemical. Preliminary studies will include both toxicity and dominant lethal tests. Tests are set up to produce 1000 control and 500 test group male progeny. Sterility or semisterility in F_1 males is determined by the sequential breeding procedure. All sterile or semisterile F_1 males are examined cytologically to confirm the presence of a translocation.

MAJOR FINDINGS AND PROPOSED COURSE: Final analysis of data from females treated with hexamethylphosphoramide indicated a possible weak dominant lethal effect in first litters. A final experiment is now being analyzed to confirm or refute this effect. Acrylamide induced high levels of dominant lethals in treated males, and a heritable translocation test is now underway on this chemical. The most important finding over the past year has been the demonstration of a dose-rate effect in the induction of dominant lethals in male mice exposed to inhaled ethylene oxide. Based on three exposure regimens, each giving a total exposure of 7200 ppm hours, increasing dose-rates resulted in increased frequencies of dominant lethals. To our knowledge this is the first demonstration of a dose-rate effect for a chemically induced genetic effect in mammalian germ cells.

Trimethyl phosphate will not be tested because of a recent manuscript reporting positive results for dominant lethals and heritable translocations. Benzene and platinol are scheduled for testing.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE: Translocations represent one of the three major categories (gene mutations, chromosomal damage, aneuploidy) of genetic events known to be associated with human genetic disease. The mouse heritable translocation test (HTT) is currently the only practicable method for detecting and quantifying induced heritable chromosomal damage in mammals. Results from the HTT provide a qualitative assessment of a chemical's potential for inducing translocations as well as quantitative data for use in genetic risk assessment efforts.

BROOKHAVEN NATIONAL LABORATORY
Upton, New York 11973
(Y01-ES-20099)

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37830
(Y01-ES-20101)

TITLE: Chromosome Aberrations and Sister-Chromatid Exchanges in Human Lymphocytes

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Michael Bender (Y01-ES-20099)
Dr. R. Julian Preston (Y01-ES-20101)

PROJECT OFFICER (NIEHS): Michael D. Shelby, Ph.D., Head
Mammalian Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: Y01-ES-20099 = \$225,890
Y01-ES-20101 = \$125,295

PROJECT DESCRIPTION

OBJECTIVES: The first objective of this project is to develop a standardized protocol by which the frequencies of chromosome aberrations and sister-chromatid exchanges can be accurately and reproducibly determined in human lymphocytes. The second objective is a better understanding of the frequencies of these cytogenetic endpoints and the variables (e.g. sex, age, race, time) that may affect their frequencies. The long range goal is to provide a step toward improving our ability to design and interpret cytogenetic studies of humans possibly exposed to genotoxic chemicals.

METHODS EMPLOYED: Lymphocyte cultures are established from heparinized whole blood samples within 24 hrs of collection. Culture medium containing 5-bromodeoxyridine is used to determine the frequency of first, second, and third division cells at harvest time (48 hrs for aberrations, 56 hrs for SCE) and to provide bromodeoxyuridine-substituted chromosomes for the scoring of SCE. For chromosome aberrations, 200 cells per individual are scored and 50 are scored for SCE.

MAJOR FINDINGS AND PROPOSED COURSE: Chromosome aberration and sister-chromatid exchange scoring was completed on the original study population of 359 employees of Brookhaven National Laboratory. Final data are being entered on the computerized data file for storage and analysis. Preliminary analysis of chromosome aberration data have not revealed any significant differences related to age, sex, race, smoking, working with solvents or radiation or taking prescription drugs. In sister-chromatid exchange analysis, the only variable that clearly affected SCE frequency was smoking. There was an indication that SCE frequencies may be affected by sex or age, but analysis of the completed data set will be necessary to confirm or refute these preliminary observations. Sampling of an extended study group to include older and younger age groups and increase the number of non-whites has commenced and scoring of slides from these new subjects is underway at both ORNL and BNL.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE:

Monitoring peripheral lymphocytes for cytogenetic damage offers one of the few available means for detecting exposures of individuals or populations to genotoxic agents. A better understanding of the variability in frequencies of chromosome aberrations and SCEs and the sources of variability will, along with standardized protocols with demonstrated interlaboratory reproducibility, permit better design and interpretation of future human cytogenetic monitoring and surveillance studies.

OAK RIDGE ASSOCIATED UNIVERSITIES
Oak Ridge, Tennessee 37830
(Y01-ES-20100)

BROOKHAVEN NATIONAL LABORATORY
Upton, New York 11973
(Y01-ES-20098)

TITLE: Evaluation and Application of an in vivo Mouse Assay for Chemically Induced Sister-Chromatid Exchanges and Chromosome Aberrations

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Alfred F. McFee (Y01-ES-20100)
Dr. Raymond Tice (Y01-ES-20098)

PROJECT OFFICER (NIEHS): Michael D. Shelby, Ph.D., Head
Mammalian Mutagenesis Group, CGTB

DATE INITIATED: September 30, 1981

CURRENT ANNUAL LEVEL: Y01-ES-20100 = \$265,188
Y01-ES-20098 = \$246,645

PROJECT DESCRIPTION

OBJECTIVES: This project is being conducted, first, to develop and assess a testing protocol for the determination of chemically induced chromosomal aberrations and sister-chromatid exchanges in mouse bone marrow cells. Second, the protocol developed will be used to test approximately 20 chemicals per year for in vivo cytogenetic effects.

METHODS EMPLOYED: B6C3F₁ male mice, 8-10 weeks old, are treated by intraperitoneal injection with the study compounds. Cell proliferation kinetics and the frequencies of chromosomal aberrations and sister-chromatid exchange are determined in bone marrow cells.

MAJOR FINDINGS AND PROPOSED COURSE: Activities in the mouse bone marrow cytogenetics projects have concentrated on the testing of chemicals selected primarily from three groups: (1) chemicals selected for NTP rodent chronic toxicity tests; (2) carcinogen-noncarcinogen pairs; and (3) chemicals of specific interest to the CGTB.

Chemicals tested or on test in these categories include:

1. N-hexane, theophylline, tetrachlorophthalic anhydride, 1,2-cis-dichloroethylene, 1,2-trans-dichloroethylene, chloroform, and bromoform
2. 4-nitro-o-phenylenediamine and 4-chloro-o-phenylenediamine, 2-(chloromethyl)pyridine and 3-(chloromethyl)pyridine, o-anthranilic acid and o-toluidine; pyrene and benzo(a)pyrene; 4-acetylaminofluorene and 2-acetylaminofluorene
3. benzoin, caprolactam, benzidine, 4-nitroquinoline-N-oxide, melphalan, 2-naphthylamine, and 2-aminobiphenyl

The staff at Brookhaven National Laboratory are currently heavily committed to the cytogenetic analysis of mice exposed by inhalation to methyl isocyanate at the NIEHS inhalation facility. These studies include both single, high-dose exposures and 4-day, multiple exposures at lower concentrations.

The emphasis of these projects will remain on the testing of NTP priority chemicals and on further defining the role of in vivo short-term tests in carcinogen predictions through the testing of carcinogen-noncarcinogen pairs.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE:

Chromosomal aberrations and sister-chromatid exchanges are both endpoints associated with the induced genetic effects of many chemical mutagens and carcinogens. As such, these endpoints are potentially important as predictors of chemical genotoxicity, particularly when conducted in whole mammals where conditions for metabolism, distribution, etc. are more reflective of the human situation than are in vitro studies. The studies as conducted provide direct evidence of genotoxic effects in laboratory mammals, an effect that can, when necessary, be compared to similar effects in exposed humans. Further, the studies are being carried out in the mouse strain used in the cancer bioassay program, and, hence, will permit a more direct comparison of induced somatic-cell genetic effects with carcinogenicity results. In addition to providing a prescreen for carcinogens, results from these tests may permit a better understanding of the relationship between induced cytogenetic effects and induced cancer.

RESEARCH TRIANGLE INSTITUTE
Research Triangle Park, North Carolina 27709
(N01-ES-25012) and (N01-ES-55078)

TITLE: Mouse Electrophoretic Germinal Mutation Test Development

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Susan E. Lewis

PROJECT OFFICER (NIEHS): Michael D. Shelby, Ph.D., Head
Mammalian Mutagenesis Group, CGTB

DATE INITIATED: December 1, 1981 (N01-ES-25012)
March 9, 1985 (N01-ES-55078)

CURRENT ANNUAL LEVEL: \$544,224

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this contract are to investigate induced mutation processes in mouse germ cells by studying cell stage sensitivities in both sexes and establishing a dose-response curve in spermatogonia using the mutagen, N-ethyl-N-nitrosourea (ENU), to characterize the electrophoretic assay using ionizing radiation as the mutagen, and to test a limited number of environmentally significant chemicals for germ cell mutagenicity.

METHODS EMPLOYED: Induced mutant frequencies are determined by treating one parent (C57B1/6J or DBA/2J), usually the male, with ENU or a test chemical and then mating to the alternate strain to obtain progeny. Blood and kidney samples are taken from the progeny and tissue preparations of these samples are subjected to starch gel electrophoresis or isoelectric focusing. After appropriate staining, the electrophoretic patterns of 32 proteins are observed on the gels and altered mobility patterns or missing bands are noted as variants. Breeding tests with the animals from which the altered proteins were obtained, along with additional electrophoretic analyses, are used to confirm the mutational basis of the variants.

MAJOR FINDINGS AND PROPOSED COURSE: The number of control loci scored during the past year exceeded 149,000 for a three-year total of 368,590. One newly arisen mutation has been recovered at the Trf locus.

Mutation induction studies in postmeiotic germ cell stages have been conducted in males and females. In both cases, ENU has been shown to induce mutations. The postmeiotic germ cells stages of males appear to yield a much lower mutant frequency than premeiotic cells. Dominant, visible mutations occurred among the mutants recovered from the treated, late germ cell stages of both sexes. A further, interesting aspect of mutants recovered from treated, late germ cell stages is that in four cases, the mutant F₁ animals were demonstrated to be somatic and/or germ cell mosaics.

The mouse model for the human condition β -Thalassemia discovered earlier in this project has been distributed to a number of laboratories for detailed studies. A description of the phenotype and heritability of this mutation was published (Skow et al., 1983, Cell 34: 1043-1052).

Tests for the mutagenicity of inhaled ethylene oxide were completed. Among 1891 progeny from ethylene oxide-treated male parents, three mutants with morphologically variant characteristics and one electrophoretic variant were recovered and demonstrated to carry heritable mutations. One morphological and one electrophoretic variant were recovered that did not breed but were shown to be translocation carriers. No variants, either electrophoretic or visible, were recovered from nearly 1400 control progeny. Thus, inhaled ethylene oxide was demonstrated to induce mutations resulting in both electrophoretically variant proteins and morphological changes inherited as dominant traits.

Experiments to complete the ENU dose response curve for spermatogonia have been completed, but final mutation frequency calculations await the outcome of heritability studies on a few variants.

Experiments are now underway to characterize the electrophoretic specific locus assay using ionizing radiation. The mutagenicity of two additional chemicals will be tested in the system over the next period of the contract.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE: This project is designed to contribute to the assessment of chemical toxicity by: 1) testing chemicals for the ability to induce mutations in mammalian germ cells, 2) providing data for use in human genetic risk estimation, and 3) contributing to an understanding of the process of induced mutations in mammalian germ cells.

LITTON BIONETICS, INC.
Kensington, Maryland 20895
(N01-ES-28036)

INVERESK RESEARCH INTERNATIONAL, LTD.
Musselburgh, Scotland
(N01-ES-28037)

TITLE: Mouse Lymphoma Cell Mutagenesis Assay

CONTRACTOR'S PROJECT DIRECTORS: Dr. Brian C. Myhr (N01-ES-28036)
Dr. Douglas McGregor (N01-ES-28037)

PROJECT OFFICER (NIEHS): William J. Caspary, Ph.D., Head
Chemical Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: September 30, 1982

CURRENT ANNUAL LEVEL: N01-ES-28036 = \$512,291
N01-ES-28037 = \$367,372

PROJECT DESCRIPTION

OBJECTIVES: The objective of this study is to assess the mutagenic activity of chemicals using forward mutation at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells. A secondary objective is to investigate the effects of changes in certain protocol elements, such as different treatment and mutant selection conditions or the composition of the exogenous metabolic activation system, on the response obtained with this assay system.

METHODS EMPLOYED: A standard protocol was employed to test for the mutagenicity of coded chemicals supplied by the NIEHS Chemical Repository. Mouse lymphoma cells (L5178Y) heterozygous at the TK locus were exposed to chemical for four hours in the absence or presence of a rat liver S9 metabolic activation system. After allowing a two-day expression period, mutagenesis was assayed as the growth of colonies in soft agar containing 5-trifluorothymidine as the selective agent. In experiments where significant increases in mutant frequency were obtained, the distributions of mutant colony sizes were obtained and analyzed. Electronically modified colony counters were used to obtain the size distributions of mutant colony populations in soft agar.

The methods for research studies were primarily the same as those used for culturing and cloning the cells in chemical testing experiments. In addition, the generation and decay of NADPH were determined spectrophotometrically at 340 nm. Osmolarity was measured with a vapor phase osmometer, and ion-specific electrodes were used to determine sodium and potassium in concentrations in culture medium.

MAJOR FINDINGS AND PROPOSED COURSE: The primary activity was the testing of coded chemicals for mutagenesis. An additional 66 compounds were tested during the year and technical reports were prepared (or are in preparation) for each chemical. Most of the mutagenic chemicals caused a preferential increase in the number of small mutant colonies, possibly indicating chromosomal rearrangements affecting the TK locus.

Studies on the S9 activation system have shown that saturating levels of NADPH are maintained during the 4-hour chemical treatment period at 37°C when 5% heat-inactivated horse serum is present in Fischer's medium and 1mM NADP and 5mM isocitrate are combined with the S9. The optimum amount of S9 was dependent on the preparation batch. Some batches of S9 showed an activation profile with a sharp maximum near 10 μ l/ml and others gave a broader profile with a maximum near 50 μ l/ml. Activation profiles for different chemical structures should be obtained for each S9 preparation in order to choose the best concentration for the screening of coded chemicals.

S9 itself was shown to be mutagenic to L5178Y cells and its mutagenic activity was greatly increased by pre-incubation at 37°C. Different lots of S9 required different amounts of pre-incubation time (1 to 3 days) to induce large increases (5- to 7-fold) in the mutant frequency after 4 hours of exposure to the cells. The mutagenic activity was observed for noninduced S9 as well as Aroclor 1254-induced S9 and was enhanced in the presence of fresh S9 mix, which included NADPH production. Primarily small mutant colonies were induced, which suggested that clastogenic substances were produced. These results indicate that S9-specific mutagenesis by test chemicals could involve chemical induction of S9 mutagenesis as well as S9 activation of the test chemical to mutagenic intermediates.

Experiments were conducted on the stringency of mutant selection using TFT in Fischer's medium and RPMI 1640 medium. The results showed that the most desirable condition for mutant selection with TFT (giving the lowest background mutant frequency in negative controls) was a medium low in phosphate ions and containing heat-inactivated horse serum. Since horse serum is known to contain nucleotide phosphorylase, which is heat labile, the most likely explanation is that TFT and phosphate ions combine via phosphorylase activity to form trifluorothymidine (which would not be selective) and ribose-1-phosphate. The selective pressure of 4 μ g/ml TFT in either medium was fully maintained for more than 4 days at 37°C when the serum was properly heat-inactivated.

Physiological limits to testing with this assay system were indicated by the effects of acid pH and high salt concentrations on the mutant frequency. As the pH was lowered to 6.5 in the presence of S9 mix, using HCl or organic acids, the mutant frequency increased sharply; very little effect was obtained in the absence of S9 mix. Alkaline pH had no effect on the mutant frequency. Also, salts such as NaCl and KF at concentrations sufficiently high to cause toxicity consistently induced increases in the mutant frequency. Fluoride ion was more effective than potassium, sodium or chloride ions. The minimum effective concentrations were approximately 8 mM fluoride, 100 mM potassium, and 230 mM sodium (including the amounts already present in Fischer's medium).

High concentrations of sucrose that caused large increases in osmolarity had no effect on toxicity or the mutant frequency. The combination of high osmolarity and high ionic strength, however, may cause instability at the TK locus. Studies on the inter-relationships of pH, ionic strength and osmolarity and their effects on the mutant frequency will continue.

A selected number of coded chemicals, already tested at the TK locus in L5178Y cells, will be tested at the HGPRT locus in CHO cells. The purpose of this

study is to determine whether any special sensitivity to chemical toxicity is occurring at the TK locus in the mouse lymphoma cells. A protocol for the CHO mutagenesis experiments is under study.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The National Toxicology Program (NTP) organizes and conducts a comprehensive inter-agency testing and research program focused on determining potential human health hazards due to environmental exposure to chemicals. This work supports these efforts by identifying the potential mutagenic activity of compounds which may be hazardous to man.

SRI INTERNATIONAL
Menlo Park, California 94025
(NO1-ES-28038)

TITLE: Evaluation of Small Colony/Large Colony Phenomenon in Mouse Lymphoma Assay

CONTRACTOR'S PROJECT DIRECTORS: Ann D. Mitchell, Ph.D.
Colette J. Rudd, Ph.D.
William F. Blazak, Ph.D.

PROJECT OFFICER (NIEHS): William J. Caspary, Ph.D., Head
Chemical Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: September 30, 1982

CURRENT ANNUAL LEVEL: \$257,275

PROJECT DESCRIPTION

OBJECTIVES: The L5178Y mouse lymphoma cell mutagenesis assay has been a useful in vitro tool for the assessment of the mutagenic potential of chemicals. One characteristic of this test is that the mutant colonies consist of large and small colonies. It has been reported that the small colonies have a high frequency of chromosomal aberrations. The objectives of this contract are to (1) investigate the cause of the bimodal size distribution of trifluorothymidine resistant (TFT^r) colonies from control and mutagen treated cultures and (2) if chromosomal damage is confirmed to be closely correlated with small colony formation, develop a cost and time efficient screening system to measure both point mutations and chromosomal damage.

METHODS EMPLOYED: Colonies have been generated in mutagenesis experiments with L5178Y mouse lymphoma cells for cytogenetic analysis and investigation of factors affecting their size distribution. The effects of selection in different culture media, time of the selection period, colony density, and length of the expression period on the size distribution have been examined. Sections of small and large TFT^r colonies 6 to 10 days old have been examined microscopically. Banded chromosome analysis has been performed on cells from 34 small and 8 large chemical mutagen induced TFT^r colonies. These cells were analyzed after culturing the colonies for 4 to 10 days after removal from agar. A new, short-term method for chromosomal analysis of colonies after only 1 to 3 days of culture has been implemented.

MAJOR FINDINGS AND PROPOSED COURSE: The recovery of small TFT^r colonies has been much more dependent on the culture conditions than the recovery of large colonies. Microscopic measurements of their diameter has shown that at the end of the selection period many were near the limits of the sensitivity of the Artek colony counter. Culture conditions that resulted in smaller average size of colonies significantly changed the number detected. Growth studies also indicated that the rate of increase in colony diameter decreased at the later times during the selection period. Examination of sections of the colonies has shown an increasing

number of necrotic cells in the center of the colonies after 7 to 8 days, with the necrotic cells appearing first in the faster-growing colonies.

Cytogenetic analyses confirmed a relationship between rearrangements involving one chromosome 11 which bears the TK locus and small colony size; i.e., aberrations involving a number 11 chromosome were found in 47% (16/34) of small colonies, whereas none were found in 8 large colonies. One large colony had a rearrangement involving chromosomes other than number 11. The majority of small colonies with cells having a rearrangement involving chromosome 11 also had cells with normal chromosomes. The most plausible explanation at this time for this mosaicism is karyotypic evolution from an abnormal number 11 chromosome to an apparently normal chromosome. Two small colonies had tandem dicentric chromosomes involving chromosome 11. These dicentrics were stable and were observed in all cells examined even after 30 cell generation in culture.

Cytogenetic studies using short-term (i.e., 1 to 3 days) culture of colonies, and selection of colonies 7 to 8 days, instead of 10 to 11 days, after cloning have been initiated. This change in protocol has been initiated in order to cytogenetically characterize the colonies as soon as possible after cloning the chemically treated cells, and to minimize the possible influence of the abundant dead cells present in colonies after 10 to 11 days' growth in cloning medium. Using this protocol, cytogenetic studies will be conducted on cells from small and large colonies from four different experimental conditions: VC-chemical, VC + chemical, TFT-chemical (solvent control), and TFT + chemical. Studies of the stability of the karyotype in cells from TFT^r colonies will also be conducted.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Recently, evidence has been accumulating on the association of cellular chromosomal abnormalities with an increased probability for tumor development. This project is designed to evaluate the possibility of obtaining information from the L5178Y mouse lymphoma cell mutagenesis assay which may reflect the relative ability of a chemical to cause chromosomal damage. If this additional knowledge can be gained with little or no additional cost, then the assay could prove to be a valuable prescreen for clastogenic chemicals.

NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH, FDA
Jefferson, AR 72079
(Y01-ES-30104)

TITLE: Coordinated Testing Project (CGT Rapid Test Response - Component 1: In Vitro Unscheduled DNA Synthesis (UDS) in Rat Hepatocyte Assay)

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Daniel Casciano

PROJECT OFFICER (NIEHS): Judson W. Spalding, Ph.D.
Cellular and Genetic Toxicology Branch

DATE CONTRACT INITIATED: 1. December 13, 1981 (Y01-ES-20077)
2. December 31, 1982 (Y01-ES-30104)

CURRENT ANNUAL LEVEL: \$150,650

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are: 1) to detect chemically-induced DNA damage/repair measured by the incorporation of labeled thymidine into cellular DNA using an autoradiographic technique; and 2) to test approximately 40 coded National Toxicology Program (NTP) chemicals for the induction of unscheduled DNA synthesis in primary rat hepatocytes. This short-term test comprises one of five test components used to characterize the genotoxic activity of chemicals.

METHODS EMPLOYED: Primary hepatocytes are isolated following an in situ perfusion of rat livers with collagenase according to a standard protocol. Isolated cells are allowed to attach to coverslips for 1-2 hours, and they are then exposed for 18-24 hours to a test chemical over an appropriate concentration range in the presence of ³H-thymidine. After incubation, the cells are processed for subsequent autoradiographic examination. The cells are stained and grains in the emulsion over the nuclei and cytoplasm are counted either visually or with an electronic counter.

MAJOR FINDINGS AND PROPOSED COURSE: A three-year contract was initiated on December 31, 1982. Forty coded chemicals have been assigned for test each year. These have included the candidate chemicals for the prechronic rodent carcinogenesis bioassay, chemicals of interest and significance to NTP experimental design groups, chemicals presently scheduled in the chronic rodent bioassay, and chemicals completed in the rodent carcinogenesis bioassay and selected for retrospective evaluation of their genetic toxicity. The results from completed assays are currently being compiled and evaluated. The scoring of the endpoint has been automated and interfaced directly with a computer to facilitate data tabulation, statistical analyses and tracking for audit. This assay is capable of identifying hepatocarcinogens or their metabolic intermediates that interact with DNA and subsequently induce DNA repair. Retrospective studies on chemicals completed in the rodent carcinogenesis bioassay permit the opportunity to distinguish between chemicals that are inducers of liver tumors and those that have "promoter" activity. The results of the chemicals tested retrospectively in this assay will be evaluated in this context.

The current contract will terminate December 31, 1985. It is expected that all tests will be completed by that date. It is the intent to initiate a new contract to continue this assay. The project will focus on: 1) the identification of hepatocarcinogens from among those chemicals of concern to NTP; 2) the improvement of the integrity of liver hepatocytes cultured in vitro; 3) the development of the UDS assay in hepatocytes from other rodent species, e.g., the mouse or hamster; and 4) the development of multiple endpoints in rodent hepatocytes for detecting genetic and cellular toxicity.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE: The Cellular and Genetic Toxicology Branch has the responsibility to provide short-term test information which can be utilized by the experimental design groups and in the ranking process for establishing the priority of chemicals to be entered into long-term carcinogenicity bioassays. The CGTB has developed a short-term test program that includes four broad classes of in vitro and in vivo short-term tests that will provide a comprehensive assessment of the capability of chemicals to effect mutation, chromosomes and DNA damage. The UDS assay in isolated rat hepatocytes is an integral part of this testing program and detects specifically the ability of chemicals to cause DNA damage and elicit a DNA repair process. This assay is useful in characterizing one mechanism by which chemicals can express genotoxic activity and potential.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20816
(N01-ES-3-5021)

SRI INTERNATIONAL
Menlo Park, California 94025
(N01-ES-3-5022)

TITLE: Salmonella Mutagenesis Testing

CONTRACTOR'S PROJECT DIRECTORS: Steve Haworth, Ph.D. (N01-ES-3-5021)
Kristien Mortelmans, Ph.D. (N01-ES-3-5022)

PROJECT OFFICER (NIEHS): Errol Zeiger, Ph.D., Supervisory Microbiologist
Environmental Mutagenesis Group, CGTB

DATE CONTRACTS INITIATED: January 15, 1983

CURRENT ANNUAL LEVEL: N01-ES-3-5021 = \$155,140.
N01-ES-3-5022 = \$188,780.

PROJECT DESCRIPTION

OBJECTIVES: The purpose of these contracts is to test environmental and commercial chemicals for mutagenicity using Salmonella typhimurium tester strains in 2 laboratories. Based on results in Salmonella chemicals will be selected for chemical analysis and further testing for other evidence of genetic toxicity.

METHODS EMPLOYED: Salmonella typhimurium strains TA97, TA98, TA100, TA1535, and TA1537 are being used to test for mutagenicity in a preincubation modification of the Ames Salmonella microsome assay. All chemicals are incubated with tester strains in suspension prior to addition of soft agar and plating for detection of induced mutants. Exogenous metabolic activation is provided by liver S-9 preparations from Aroclor 1254-induced Sprague-Dawley rats and Syrian hamsters. All chemicals are tested under code at 5 doses, in triplicate. The test protocol has recently been modified to initially test in TA98 and TA100. If a positive result is obtained in at least one of these strains, the other strains are not used. If the chemical is negative in these two strains, it is tested in strains TA97, TA1535, and TA1537. Also, all chemicals are retested at least one week following the first test. Results are being entered directly into a minicomputer for transfer to the Cellular and Genetic Toxicology Branch data-base system.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratories have tested 204 samples (196 unique chemicals) during the past year and it is anticipated that an additional 200 samples will be tested this year. Information and data on specific chemicals are being provided to government personnel and the private sector on request. Results of these tests are, and will continue to be routinely published in peer-reviewed scientific journals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These contracts allow the NTP to rapidly screen large numbers of chemicals for mutagenicity in a relatively short time and at relatively low cost. Mutagenicity in this system correlates strongly with carcinogenicity and heritable mutations in rodents. The results of these Salmonella tests will be used to assist in decisions regarding chemicals to be tested in sub-chronic and chronic toxicological tests, as well as other genetic toxicity tests.

PUBLICATIONS

Mortelmans, K., Haworth, S., Speck, W., and Zeiger, E.: Mutagenicity testing of Agent Orange components and related chemicals. Toxicol. Appl. Pharmacol. 75: 137-146, 1984.

Zeiger, E., and Haworth, S.: Tests with a preincubation modification of the Salmonella/microsome assay. In Ashby, J., de Serres, F.J., Draper, M., Ishidate, M., Jr., Margolin, B., Matter, B., and Shelby, M.D. (Eds.): Collaborative Study of Short-Term Tests for Carcinogens. Amsterdam, Elsevier/North Holland, 1985, pp. 187-199.

Zeiger, E., Haworth, S., Mortelmans, K., and Speck, W.: Mutagenicity testing of di(2-ethylhexyl)phthalate and related chemicals in Salmonella. Environ. Mutag. 7: 213-232, 1985.

UNIVERSITY OF WISCONSIN
Madison, Wisconsin 53707
(N01-ES-3-5028)

BROWN UNIVERSITY
Providence, Rhode Island 02912
(N01-ES-3-5027)

TITLE: Drosophila Mutagenesis Testing

CONTRACTOR'S PROJECT DIRECTOR: Ruby Valencia, Ph.D. (N01-ES-3-5028)
Stanley Zimmering, Ph.D. (N01-ES-3-5027)

PROJECT OFFICER (NIEHS): James Mason, Ph.D., Geneticist
Environmental Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: N01-ES-3-5028: September 28, 1983
N01-ES-3-5027: September 28, 1983

CURRENT ANNUAL LEVEL: N01-ES-3-5028 = \$185,836
N01-ES-3-5027 = \$191,359

PROJECT DESCRIPTION

OBJECTIVES: The purpose of these contracts is to test a total of 40 environmental and commercial chemicals per year for mutagenicity using Drosophila melanogaster tester strains in two laboratories. Substances which are found to induce sex-linked recessive lethal mutations in Drosophila will be tested for the ability to induce chromosome translocations. The use of Drosophila as a test organism will allow us to characterize the types of mutations induced in germ cells in vivo by chemicals which have been shown to be mutagenic in in vitro test systems. The information will be used in combination with results from other tests to evaluate the overall genotoxic effects of chemicals.

METHODS EMPLOYED: Standard sex-linked recessive lethal and reciprocal translocation tests in Drosophila melanogaster are being used to test for mutagenicity. Chemicals are administered by feeding and the recessive lethal test is performed. If the results are negative, the test is repeated after injection of the chemical. If the results are again negative, the chemical is considered nonmutagenic in Drosophila. If the results are positive, the chemical is tested in the reciprocal translocation test using the means of administration which gave the positive result. In the translocation test, sperm are stored to enhance the ability to recover chromosome breaks induced by the chemicals.

MAJOR FINDINGS AND PROPOSED COURSE: A series of 29 chemicals has been tested using larval feeding as the route of administration in order to compare this procedure with others that have been used in the past. This comparison is of interest because larvae have a different array of metabolic activation enzymes than adults and, therefore, may be able to activate a different set of promutagens. The results indicate that the larval feeding technique, as applied in these studies, does not adequately complement the adult treatment procedures when aromatic amines and polycyclic hydrocarbons are assayed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These contracts will allow the NIEHS to confirm in vitro mutagenicity results in a whole animal in vivo test system. Drosophila is capable of activating promutagens by means of an enzyme system similar to the mammalian activation system.

PUBLICATIONS

Zimmering, S., Mason, J.M., Valencia, R., and Woodruff, R.C.: Chemical mutagenesis testing in *Drosophila*. II. Results of 20 coded compounds tested for the National Toxicology Program. Environ. Mutagen. 7: 87-100, 1984.

Valencia, R., Mason, J.M., Woodruff, R.C., and Zimmering, S.: Chemical mutagenesis testing in *Drosophila*. III. Results of 48 coded compounds tested for the National Toxicology Program. Environ. Mutagen. 7: 325-348, 1985.

Yoon, J., Mason, J.M., Valencia, R., Woodruff, R.C., and Zimmering, S.: Chemical mutagenesis testing in *Drosophila*. IV. Results of 45 coded compounds tested for the National Toxicology Program. Environ. Mutagen. 7: 349-368, 1985.

Woodruff, R.C., Mason, J.M., Valencia, R., and Zimmering, S.: Chemical mutagenesis testing in *Drosophila*. V. Results of 53 coded compounds tested for the National Toxicology Program. Environ. Mutagen., 1985, in press.

UNIVERSITY OF KENTUCKY
Lexington, Kentucky 40503
(N01-ES-3-5032)

TITLE: Chemically Induced Transposition in *Drosophila*

CONTRACTOR'S PROJECT DIRECTOR: Dr. Christopher Osgood

PROJECT OFFICER (NIEHS): James M. Mason, Ph.D., Geneticist
Environmental Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$157,038

PROJECT DESCRIPTION

OBJECTIVES: It has been observed that a large proportion, possibly a majority, of spontaneous mutations in *Drosophila* result from the insertion of transposable elements into or near the affected genes. The purpose of this contract is to determine whether transposition of mobile genetic elements can be induced in *Drosophila* by chemical treatments, and to develop an assay for chemically-induced transposition that can be used to screen potential mutagens.

METHODS EMPLOYED: Two steps are required to demonstrate that transpositions can be caused by chemicals. In the first step individuals carrying mutations known to result from transposons inserted into specific genes are treated with a chemical mutagen and the progeny of these individuals are examined for reversion of the mutations that were caused by the insertion. These progeny can also be examined for the appearance of new visible mutations at the same time. In the second step, DNA from the exceptional progeny (either the revertants or the progeny bearing new mutations) is examined on Southern blots to ask whether the reversions were caused by an excision of the transposable element that was originally present or whether the new mutations were due to an insertion of DNA into the gene that was mutated.

MAJOR FINDINGS AND PROPOSED COURSE: A total of 58 ethyl methanesulfonate- or ethyl nitrosourea-induced mutations at the RpII locus were examined for changes in their restriction pattern on Southern blots. Three of the mutations exhibited such changes, but they were all found to be small deletions of material from within the gene. No insertions were found.

Screens are now underway to collect revertants resulting from excisions of transposable elements. Several genetically stable partial reversions of w^a (an insertion of copia in the w locus) have been recovered. These mutations are being analyzed at the molecular level to ask whether insertion sequences have been excised. The evidence so far suggests that excision of the copia element occurs in some tissues (but not the germ line) in succeeding generations to produce mosaics containing Southern banding pattern for both the copia insert and the wild type in the same fly.

At this point it is still not known whether chemicals induce transposition of mobile genetic elements directly. Given the almost total lack of knowledge in this area, it is not clear which chemical classes or which transposable elements should be used. Plans are to treat individuals carrying a number of different transposable elements inserted into known, cloned genes with a wide variety of chemicals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM AT THE INSTITUTE: It has been demonstrated in *Drosophila* that a large proportion of apparently spontaneous mutations, including mutations recovered from natural populations, are the result of insertion of transposable elements. Transposition of these elements is very rare, however, under normal laboratory conditions. It is not known to what extent transposition is a cause of spontaneous mutation in mammals, nor is it known to what extent exposure to chemicals induces transposition. This project is designed to answer the latter in *Drosophila* and, if chemicals are found to induce transpositions, to design and develop an assay for chemically-induced transposition which will be used as part of the Cellular and Genetic Toxicology Branch mutagen screening program.

U.S. DEPARTMENT OF AGRICULTURE
Western Regional Research Center
Berkeley, CA 94710
(Y01-ES-40113)

TITLE: Evaluation of the Mouse Erythrocyte Micronucleus Assay as an Adjunct to Rodent Toxicity Tests

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. James T. MacGregor

PROJECT OFFICER (NIEHS): Michael D. Shelby, Ph.D., Head
Mammalian Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: October 1, 1983

CURRENT ANNUAL LEVEL: \$40,539

PROJECT DESCRIPTION

OBJECTIVES: The primary objective of this project is to evaluate the utility of the peripheral erythrocyte micronucleus test for cytogenetic damage as an adjunct to prechronic toxicity studies in mice. This is being approached by comparison of the frequency of micronucleated peripheral erythrocytes in mice exposed to test chemicals for 90 days or two years to micronucleus frequencies in the bone marrow cells of mice treated with single, high doses of the same chemicals.

METHODS EMPLOYED: Blood smears (slides) from male and female mice are obtained from NTP testing laboratories conducting 90-day or two-year toxicity studies. Slides are stained with Wright-Giemsa, and the frequency of micronucleated normochromatic erythrocytes is determined using 1000x magnification. A minimum of 12,000 cells (2000 cells x 6 animals) are scored at each dose. Acute, high-dose tests will be conducted using B6C3F₁ mice. Exposure will be by intraperitoneal injection or gavage with scoring carried out as in 90-day exposures.

MAJOR FINDINGS AND PROPOSED COURSE: Micronucleus data were obtained on three new chemicals and additional data were obtained on three chemicals previously studied. A manuscript on micronucleus induction by benzene was accepted for publication in Mutation Research. Evaluation of blood smears from the NTP chronic rodent assay of propylene showed no elevation of micronucleus frequencies. There was no evidence of micronucleus induction by tetrahydrocannabinol following 90-day exposure of male and female mice. Negative results were also obtained from 1,2-epoxybutane treated mice following two-year exposures.

The most notable accomplishment of the past year was the development of a semi-automated scoring method that employs a light microscope in conjunction with an automated stage control operated by a computerized image analyzer. Micronucleated erythrocytes are visually scored. Computer memory of the positions of the fields scored are then used to return to those fields and estimate cell counts through the image analyzer and a TV screen. This new methodology permits the scoring of approximately ten times the number of cells scored by the previous manual method without an increase in time or effort.

It is proposed to continue the effort to assess micronucleus frequencies in blood smears from 90-day and two-year exposed animals and to compare these with results from acute dose, bone marrow micronucleus studies. These studies may yield valuable information on the potential of this assay to discriminate between carcinogen-noncarcinogen structurally analogous pairs of chemicals, both of which have exhibited genetic toxicity in in vitro assays.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Sufficient evidence of a link between the genotoxic and carcinogenic properties of chemicals exists to justify the use of genotoxicity data in the evaluation of a chemical's potential carcinogenicity. Determination of a genetic effect in the same animals and by the same routes of exposure and dose levels may provide the more relevant information than in vitro studies. This assay system may provide that type of data from the prechronic toxicity studies and contribute valuable information to the decision of whether or not to pursue a chronic toxicity study.

OAK RIDGE NATIONAL LABORATORY
(Department of Energy)
Oak Ridge, TN 37830
(Y01-ES-40118)

TITLE: Mouse Endogenous Retroviral Long Terminal Repeat Elements for Studying Gene Transposition in Environmental Carcinogenesis

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Wen K. Yang

PROJECT OFFICER (NIEHS): Raymond W. Tennant, Chief
Cellular and Genetic Toxicology Branch

DATE CONTRACT INITIATED: January 10, 1984

CURRENT ANNUAL LEVEL: \$339,187

PROJECT DESCRIPTION

OBJECTIVES: The project addresses the important question of whether endogenous retrovirus and related proviral elements in a murine model system are functionally transposable elements which may be potential targets of environmental genotoxic agents. The conceptual framework for this research effort is based on the well known role of retroviruses in insertional mutagenesis and carcinogenesis and the structural analogy between retroviruses and transposable elements. The mouse genome contains many related families of endogenous provirus and proviral-like elements that are likely to be involved in transposition or other events related to genetic damage. This project focuses on characterizing the structure and function of a unique class of proviral-like elements that are distantly related to murine leukemia virus.

METHODS EMPLOYED: Recombinant DNA cloning of LTR and Insertion Sequence 23 (IS23) containing fragments from Balb/c and RFM/Un mouse DNA has provided a variety of endogenous proviral-like elements. These clones are being characterized by restriction enzyme mapping and nucleotide sequence analysis. The inheritance of these elements are being studied by a comparative analysis of the integration site in various strains and subspecies of the mouse.

MAJOR FINDINGS AND PROPOSED COURSE: The proviral unit designated as IS23 was found to contain a repeated sequence reiterated approximately 200 times in the mouse genome. The IS23 provirus is integrated within a region that is repeated approximately 10 times. The IS23 probe exhibits a mouse strain specific DNA restriction fragment pattern and unexpectedly recognizes a male specific fragment. Evidence that this male specific IS23 fragment is lost from certain chemically transformed NIH-3T3 cell clones and from tumors arising from chemically transformed cells will be confirmed and extended in the coming year. Solitary LTR structures have also been isolated from leukemias and their role in leukemogenesis will be examined by identifying their genomic location with respect to cellular oncogenes. Finally, an experimental transposition model system will be constructed by introducing molecular clones of these proviral elements into human or other species cells that do not have an endogenous background of related sequences.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Identification of mammalian transposable elements and their role in mutagenesis and carcinogenesis is of considerable importance in contemporary biomedical research. The information gained from research on retroviral related elements will benefit the long-term goal of developing reliable in vitro tests to measure DNA transposition as a potentially significant genetic endpoint. The ability to detect such events in a sensitive and precise manner and the ability to identify environmental agents with the potential to cause such effects are fundamental goals of the CGTB/NTP/NIEHS.

NATIONAL INSTITUTE OF OCCUPATIONAL SAFETY & HEALTH
Cincinnati, Ohio 45226
(Y01-ES-40121)

TITLE: In Vitro Tests for Workplace Co-carcinogens

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. J. Bohrman

PROJECT OFFICER (NIEHS): Robert Langenbach, Ph.D., Head
Metabolic Activation Group, CGTB

DATE CONTRACT INITIATED: September 30, 1982 (Y01-ES-20094)
March 30, 1984 (Y01-ES-40121)

CURRENT ANNUAL LEVEL: \$171,000

PROJECT DESCRIPTION

OBJECTIVES: The objective of this research is to further develop and validate an in vitro assay for tumor promoters and then to test (coded) up to 40 chemicals for which the NTP has carcinogenesis data and in vitro genetic toxicity data.

METHODS EMPLOYED: The system being studied is the V79 cell metabolic cooperativity assay developed by Trosko and colleagues. Chinese hamster V79 cells which are resistant to 6-thioquanine (6-TG) are co-cultivated with V79 cells sensitive to 6-TG. In the co-cultivation, when metabolic cooperativity exists, the 6-TG resistant cells are killed and their cloning efficiency decreases. In the presence of chemicals with promoting activity, metabolic cooperativity is decreased and an increased number of 6-TG resistant colonies survive.

MAJOR FINDINGS AND PROPOSED COURSE: Two laboratories under NIOSH and NTP's supervision are looking at the effect of varying basic parameters in the assay. These parameters include: pH effects, optimum Ca^{++} levels, optimum time between addition of test chemical and 6-TG, required duration of the assay, and statistical approaches. With the developed protocol, up to 40 coded chemicals with an NTP data base will be tested. The general ability of the assay to distinguish between carcinogens and noncarcinogens will be determined. Furthermore, the assay will be studied for its ability to identify carcinogens which act only by promoting mechanisms as suggested by their lack of genetic toxicity in vitro. The data from the metabolic cooperation assay will be analyzed in combination with carcinogenicity and genetic toxicity results.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Most short-term assays for chemical carcinogens are based on the induction of genetic damage. Therefore, chemicals which cause or promote cancer cells by nongenetic mechanisms are not readily detected in short-term assays currently used by the NTP. The development of an assay which detects such chemicals would greatly enhance the spectrum of carcinogens which short-term tests detect.

DUKE UNIVERSITY MEDICAL CENTER
Durham, North Carolina 27710
(N01-ES-55091)

TITLE: Task 1: Development of Human Cell Assay Systems for Genetic Toxicity

CONTRACTOR'S PROJECT DIRECTORS: Dr. S. Strom
Dr. G. Michalopoulos

PROJECT OFFICER (NIEHS): Robert Langenbach, Ph.D., Head
Metabolic Activation Group, CGTB

DATE CONTRACT INITIATED: May 1, 1985

CURRENT ANNUAL LEVEL: \$187,626

PROJECT DESCRIPTION

OBJECTIVES: The project is a three-year study to develop and utilize a cell-mediated system where freshly isolated human hepatocytes are used for metabolic activation and co-cultivated human target cells are used to detect genetic damage. The overall objective is to determine the utility of a human cell-mediated system in screening for genotoxic agents. Some specific objectives are: (1) analysis of individual variation of carcinogen activation by human hepatocytes; (2) measurement of UDS in the human liver cells and of mutation and SCE induction in human fibroblast for four uncoded and 12 coded chemicals; (3) comparison of human hepatocytes and rat hepatocytes for metabolic activation and human fibroblast and V79 cells as genetic targets; (4) improve freezing techniques for liver cells; and (5) compare human liver cells and human S9 for metabolic activation in genetic toxicity assays.

METHODS EMPLOYED: Human liver tissue is obtained from organ donors or surgical specimens and perfused to obtain isolated, intact, viable hepatocytes. The hepatocytes are then co-cultivated with human fibroblast in the presence of the chemical and the genetic endpoints UDS, SCE, and mutation measured. Metabolite analysis of the chemicals is done by HPLC.

MAJOR FINDINGS AND PROPOSED COURSE: Initial experiments will improve techniques for obtaining viable human hepatocytes and use the hepatocytes in a human cell-mediated system to determine the genetic effects of aflatoxin B₁, dimethylnitrosamine, acetylaminofluorene, and benzo(a)pyrene. The metabolism and metabolic activation to genotoxins of these carcinogens in hepatocyte preparations from different humans will be compared. Early studies will also focus on improving techniques to freeze hepatocytes for subsequent use in genotoxicity assays.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The study addresses the problem of utilizing human cells in determining the genetic toxicity of chemicals. Because species differences in response to chemical carcinogens exist, it is useful to determine the effects of chemicals in human cell systems, in addition to rodent cell system, to better predict human health hazard. The long-term outcome of the study will be a clearer understanding of the role of human cells in short-term assays.

UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
Chapel Hill, North Carolina 27514
(N01-ES-55092)

GENTEST LIMITED PARTNERSHIP
Woburn, Massachusetts 01801
(N01-ES-55093)

TITLE: Task II: Development of Human Cell Assay Systems for Genetic Toxicity

CONTRACTOR'S PROJECT DIRECTORS: Dr. D. Kaufman (N01-ES-55092)
Drs. C. Crespi and B. Pennman (N01-ES-55093)

PROJECT OFFICER (NIEHS): Robert Langenbach, Ph.D., Head
Metabolic Activation Group, CGTB

DATE CONTRACT INITIATED: May 1, 1985

CURRENT ANNUAL LEVEL: (N01-ES-55092) = \$252,810
(N01-ES-55093) = \$488,292

PROJECT DESCRIPTION

OBJECTIVES: The project is a three-year study to develop and utilize a human cell system for the detection of genotoxic chemicals in which metabolic activation of the chemicals and measurement of genetic endpoints can be accomplished in the same cells. As most cell systems used in genetic toxicity systems have lost varying degrees of metabolic capability, a primary objective is to improve the metabolic capacity of the cells so that more useful, reliable, and sensitive human cell systems are available for short-term testing.

METHODS EMPLOYED: Human lymphoblastoid cells will be used in the studies and attempts made to improve metabolic capabilities by fusion with metabolically active human cells, transfer of specific chromosomes, or selective cloning. Genetic endpoints measured will be mutations, DNA repair, and chromosome aberrations.

MAJOR FINDINGS AND PROPOSED COURSE: Initial experiments will determine which classes of chemical carcinogens the lymphoblastoid cells cannot metabolize. Metabolic capability will be assessed by induction of genetic endpoints and by HPLC identification of metabolites produced. Chemicals which are not metabolized by the parent cell line will be identified and attempts made to modify metabolic capability. Experiments to fuse the lymphoblastoid cells with established human cell lines of known metabolic capacity (e.g., Hep G2 or endometrial cells) and select for hybrids with enhanced metabolic capability. Alternatively, selective cloning of cells or use of cell sorting in the presence of the test chemical to identify cells within the mass population with the ability to metabolize the chemical will be employed. In later studies, 12 (UNC) and 30 (Gentest) coded chemicals will be tested in the metabolically altered human cell system for genotoxic effects.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Most cells used in vitro to assess the genetic toxicity of a chemical have lost the metabolic capability they possessed in vivo. The present study addresses the uses of human cells in genetic toxicity assays and simultaneously searches for methodologies to enhance in vitro metabolic capability. The development of

cells in which both metabolism and measurement of genetic endpoints can be accomplished should yield more reliable and sensitive cell systems for short-term tests. Furthermore, the use of human cells could produce systems which are more predictive of hazard to humans.

BROWN UNIVERSITY
Providence, Rhode Island 02912
(N01-ES-5-5095)

UNIVERSITY OF KENTUCKY
Lexington, Kentucky 40506-0225
(N01-ES-5-5106)

TITLE: Validation and Testing of Chemicals in a *Drosophila* Aneuploidy Detection System

CONTRACTOR'S PROJECT DIRECTOR: Stanley Zimmering, Ph.D. (N01-ES-5-5095)
Christopher Osgood, Ph.D. (N01-ES-5-5106)

PROJECT OFFICER (NIEHS): James M. Mason, Ph.D., Geneticist
Environmental Mutagenesis Group, CGTB

DATE CONTRACT INITIATED: N01-ES-5-5095: March 1, 1985
N01-ES-5-5106: June 1, 1985

CURRENT ANNUAL LEVEL: N01-ES-5-5095 = \$184,330
N01-ES-5-5106 = \$144,382

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this contract is to develop a test system in *Drosophila* for screening environmental chemicals for their ability to induce aneuploidy, i.e., changes in chromosome number. The use of a test for aneuploidy will allow the identification of chemicals which induce certain types of chromosomal aberrations but may not be identified as mutagenic in the standard short term mutagenesis test systems now in use.

METHODS EMPLOYED: Standard genetic manipulations are employed utilizing well characterized X-linked mutants and chromosomal aberrations in *Drosophila melanogaster*. The proposed assays for aneuploidy involve feeding test chemicals to female larvae, mating these females to tester males and scoring for the occurrence of progeny resulting from changes in chromosome number. Each chemical will be tested at 2-3 doses.

MAJOR FINDINGS AND PROPOSED COURSE: Three separate strains have been established that will allow aneuploid progeny to be recognized in a one-generation screen in which half of the regular progeny die. These three strains are being compared for sensitivity and reliability by treating each in parallel with a number of positive control compounds. The compounds have been chosen to represent different classes of chemicals known to interact with the mitotic/meiotic apparatus. The results from these tests will be used to determine the strain that will be used in future assays for chemically-induced aneuploidy in *Drosophila*. As soon as the appropriate strain is chosen, testing of known positive and negative compounds will begin in a dual laboratory study to evaluate the test system.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A large fraction of spontaneous abortion in humans and certain serious genetic diseases (e.g., Down's syndrome) are caused by aneuploidy. A few chemicals are known to induce aneuploidy; however, there is no fast, reliable, well-defined developed test to detect such chemicals on a large scale. This project is designed to develop a test system to be used as part of the Cellular and Genetic Toxicology Branch mutagen screening program. The use of a test for aneuploidy will allow the Program to identify chemicals which induce certain types of chromosomal aberrations that would not be identified as mutagenic in other mutagenesis test systems.

CHEMICAL PATHOLOGY BRANCH

CHEMICAL PATHOLOGY BRANCH
Summary Statement

Mission: During FY 85 the Chemical Pathology Branch continued its three major functions: (1) support of the National Toxicology Program (45%); (2) support of intramural research (30%); and (3) independent research (25%).

Section Work Areas: Histology and Electron Microscopy, Tumor Pathology, Toxicologic Pathology, Experimental Pathology, and Laboratory Animal Management.

Staffing: The Chemical Pathology Branch consists of 11 comparative pathologists, 1 laboratory animal veterinarian, 12 technicians, 3 secretaries, and 1 stay-in-school student. Dr. Roger Alison, formerly of Huntingdon, England, joined the Branch in February 1985. Dr. Linda Uraih, formerly of International Research and Development Corporation (IRDC), joined the Branch in March 1985. Dr. Steven Stefanski, having recently completed a pathology residency and masters program at Michigan State University, joined the Branch in April 1985. Dr. Kunitoshi Mitsumori, formerly of the National Institute of Environmental Toxicology, Tokyo, Japan, joined the Branch in May 1985. Dr. Michael Elwell, formerly of Walter Reed Army Institute of Research (transferred from the Army to the Public Health Service), joined the Branch in July 1985. Dr. Kenneth Pierce of Texas A & M University, and Dr. Jeffrey Wilson of Sandoz, Basel, Switzerland, are working in the Branch as National Institutes of Health Guest Workers.

Accomplishments:

1. Management of Quality Assurance Program for the National Toxicology Program - During FY 85 the Chemical Pathology Branch continued responsibility for evaluating the quality of pathology conducted in bioassays performed by the NTP. This included the review of 14 sub-chronic, 19 interim sacrifices, and 21 chronic bioassays during the first nine months of FY 85 (Tables 1, 2, and 3).
2. Establishment of the NTP Archives in Research Triangle Park adjacent to the NTP - The move from Rockville, Maryland, was accomplished in November 1984 and the "downtime" for the Archive was less than one month. Over 300 studies and 5.5 million slides are now available to NTP and approved persons on an easily accessible computerized system.
3. Implementation of TDMS - During FY 85 the Toxicology Data Management System (TDMS) was implemented in 3 additional contractor laboratories (total of 11) and 4 interagency agreement sites. There are presently 162 chemicals on TDMS and all new starts will be put on the system. A one-day meeting of advisors reviewed the pathology nomenclature code table and added approximately 40 new terms to the computer system.
4. Research Programs - During FY 85 the responsibility for laboratory animal management was transferred to the Chemical Pathology Branch. The laboratory animal veterinarian has a strong interest in pathology and some Branch pathologists have dual boards (Lab Animal Medicine and Pathology) which will allow a natural collaboration between these two closely related disciplines.

5. During FY 85 the Branch placed increasing emphasis on the quality of pathology data and meeting GLPs. A total of 30 studies were audited and the pathology portion reviewed by the Branch. Three major changes were instituted to improve and check the Pathology data. First, all pathology data at the end of a 2-year study is now subjected to a pre-QA data review while slides, blocks, and wet tissues are still at the laboratory. If discrepancies, duplications or unexplained data is found, the pathology tables are returned to the laboratory. Once the data is acceptable, the pathology material and data is submitted to the Archives. The second new step is a slide/block match-up, animal I.D. check, and a check for untrimmed lesions by the Archives. If unacceptable, the data is returned to the laboratory for corrections. If acceptable, it proceeds to the pathology QA. The third new step is to include the original pathologist in the Pathology Working Group (PWG) review. These three new steps have resulted in more prompt resolution of problems and fewer post-PWG unresolved pathology issues.
6. Research Programs - Studies in support of the National Toxicology Program included:
 - a. international meeting and working group on proliferative lesions of the Brain in Man and Animals co-sponsored jointly by NTP and Duke University Medical School.
 - b. modification of the reduced pathology protocol to include slide preparation but "read downs" for only required tissues. This preserves most of the savings in cost but is accomplished more quickly.
 - c. organizing an international symposium on ovarian tumors in man and animals.
 - d. development of a classification scheme and study set for ovarian tumors in rats and mice.
 - e. instituting cooperative agreements between Dartmouth Medical School, Northwestern Medical School, and the University of Missouri to evaluate the significance of dietary oils and proliferative exocrine pancreatic lesions found in male rats.
 - f. evaluate the effect of sampling on the incidence of pancreatic lesions in vehicle and untreated male and female F344 rats.
 - g. completed and submitted for publications the evaluation of the strain A mouse lung adenoma assay in evaluating the carcinogenic potential of chemicals.
 - h. evaluate the use of immunoperoxidase techniques for identifying cell types in mouse lymphomas.
 - i. transplantation of induced rodent tumors in several NTP studies. This has allowed a better definition of the biological behavior of cholangiofibrosis/cholangiocarcinoma lesions.

- j. ultrastructural evaluation of the nasal turbinate in control and methyl isocyanate studies.
 - k. evaluation of forestomach lesions in methyl bromide studies utilizing stop studies.
 - l. evaluation of the salivary sarcomas in the chlorendic acid NTP study.
 - m. review and classification of the proliferative gall bladder lesions found in mice.
 - n. review of naturally occurring chordomas in F344 rats.
 - o. institution of a modified classification scheme for proliferative hepatocellular lesions in the rat. This is based upon classic pathology terminology utilizing hyperplasia, adenoma, and carcinoma and will be used by NTP pathologists for two years at which time the results will be re-evaluated.
 - p. use of nuclear magnetic resonance (NMR) to assess liver function in intact animals.
 - q. preparation of a hematology atlas for peripheral blood and bone marrow of the F344 rat.
 - r. use of nuclear magnetic resonance in cooperation with Duke University to detect naturally occurring and induced tumors in rats.
 - s. development of whole body 5-8 micron histology sections for rats for use in NMR studies.
 - t. revision of the NTP statement of work to include a more thorough histopathologic evaluation of acute and 14-day repeated dose toxicologic studies.
7. Research Program - Independent studies and collaborative efforts with other laboratories in TRTP/NTP.
- a. mechanism of the dioxin induced myelotoxicity and immunotoxicity in mice.
 - b. myelotoxicity of the class of propylene glycols.
 - c. validation, development, and quality review of clinical pathology and chemistry in NTP studies.
 - d. investigation into the two-hit hypothesis of carcinogenesis utilizing the rat liver model.
 - e. tissue collection from NTP carcinogenicity studies as well as from in-house studies for identification on oncogenes.

- f. publication of a book chapter on pathology QA procedures for toxicity and carcinogenicity studies.
- g. publication of a book chapter on history, naturally occurring lesions in B6C3F1 mouse and F344 rat and comparison with other strains used in toxicology.
- h. evaluation of immunoperoxidase techniques for murine hematopoietic tumors and thyroid hormones
- i. evaluation of soft-plastic techniques for microscopic examination of muscle
- j. development of histochemical methods for fiber typing of striated muscle
- k. evaluation of subchronic, interim sacrifice, and chronic toxicologic studies of furan induced liver disease.
- l. successful transplantation of furan induced cholangiocarcinomas to recipient F344 rats
- m. special study of 3,3'-dimethylbenzidine induced basal cell hyperplasia of the skin
- n. ultrastructural evaluation of 3,3'-dimethylbenzidine induced hepatocellular hypertrophy in the rat
- o. studies on interstitial and pyelonephritis of the mouse and renal infarction in the mouse and rat
- p. evaluation of the prechronic toxicity of furfural and furfuryl alcohol
- q. study of the subchronic dermal toxicity of vinylcyclohexene diepoxide in rats and mice
- r. urinary bladder and kidney lesions produced in Fischer 344/N rats and B6C3F1 mice after 13 weeks of 2,2-bis(bromomethyl)-1,3-propanediol administration
- s. subchronic toxicity of propantheline bromide in Fischer 344/N rats and B6C3F1 mice
- t. study of the hepatic lesions induced by chlorowax 40 in F344/N rats
- u. study of the neuropathology of 13-week toxicity of orally administered sodium azide to F344/N rats.

8. Support of the Intramural Research Program - A great deal of support was provided in support of the Laboratory of Reproductive and Developmental Toxicology, Laboratory of Pulmonary Function and Toxicology. Lesser support was provided to Laboratory of Genetics, Laboratory of Genetic Mutagenesis, Laboratory of Behavioral and Neurological Toxicology, Laboratory of Pharmacology and Laboratory of Molecular Biophysics.

a. Laboratory of Reproductive and Developmental Toxicology

- (1) Dr. J. McLachlan - consultations on lesions found in mice exposed in utero to diethylstilbestrol (DES) and related compounds
- (2) Dr. J. McLachlan - electron microscopy on CD-1 mouse fetal genital tract culture after prenatal treatment with DES
- (3) Drs. Toshimori and Hedger - effects of anti-cancer drugs on the immature testes - electron microscopy
- (4) Dr. J. McLachlan - electron microscopy and special histologic techniques in the study of the effect of DES on the developing and adult reproductive tract
- (5) Dr. O'Brien - electron microscopy support - animal testes.
- (6) Dr. Tomooka - electron microscopy support - tissue culture

b. Laboratory of Pharmacology

- (1) Dr. C. Schiller - effects of TCDD on gut, liver, pancreas and lungs of rats, electron microscopy support
- (2) Dr. B. Fowler - several projects involving EM support of studies on the ultrastructural effects of heavy metals on the kidney and liver
- (3) Dr. M. King - electron microscopy support - liver
- (4) Ms. T. Devereaux - ultrastructural evaluation of rabbit lung cells.

c. Laboratory of Pulmonary Function and Toxicology

- (1) Dr. R. DiAugustine - neuroendocrine epithelial cells of the guinea pig upper respiratory tract - electron microscopy support
- (2) Dr. G. Hook - studies on the composition and ultrastructure of abnormal tubular myelin assembly in the lungs of patients with pulmonary alveolar proteinosis - electron microscopy support

- (3) Dr. A. Brody - deposition and translocation of inhaled asbestos - electron microscopy support
- (4) Dr. A. Brody - deposition and translocation of tracheal crystalline silica - electron microscopy support
- (5) Dr. G. Hook - comparison of acute morphological pulmonary changes with enzyme profiles in pulmonary lavage material - histopathology evaluation.

d. Laboratory of Molecular Biophysics

e. Statistics and Biomathematics Branch

- (1) Dr. J. Haseman - updated and prepared for publication the current historical tumor rates in the F344 rat and the B6C3F1 mouse
- (2) Dr. J. Haseman, Dr. R. Melnick (CGTB) - urolithiasis and bladder carcinogenicity of melamine in rodents
- (3) Dr. J. Haseman, Dr. K. Abdo (CGTB), Dr. M. Dieter (CGTB) - carcinogenesis bioassay of propyl gallate in F344 rats and B6C3F1 mice.

- 9. Data Audit Reviews - A data audit procedure was in place in 1985 for recently completed NTP technical reports and for all future reports coming to peer review. During 1985, 30 studies (Table 4) were subject to data audit. The pathology portion included verification of carcass identification, review for gross-microscopic correlation, slide/block match-up, review of slide label identification, and verification of accurate recording of pathology data on individual animal data records. This process was done by contract personnel, NTP personnel, and the pathology portion of the audit reports was reviewed by the Chemical Pathology Branch.

TABLE 1
SUBCHRONIC STUDIES REVIEWED DURING FY 1985

4-Chloro-2-Nitroaniline

Tripelennamine

Methylbromide

Psoralen IV

Carisoprodol

2-Mercaptobenzimidazole

Pyrilamine

Triprolidine

Methylphenidate Hcl

Hexachlorocyclopentadiene

4,4-Thiobis(6-t-Butyl-m-Cresol)

D-Alpha-Tocophenyl Acetate

Δ^9 -Tetrahydrocannabinol

TABLE 2

INTERIM SACRIFICE STUDIES REVIEWED DURING FY 1985

3,3'-Dimethoxybenzidine
3,3'-Dimethoxybenzidine (Review of early deaths)
Tris(2-Chloroethyl)Phosphate
Titanocene Dichloride - 15 months
Vinyl Cyclohexene Diepoxide - 15 months
Polysorbate 80 - 15 months
H.C. Yellow 4 - 15 months
Pentachloroanisole - 9 months
C.I. Pigment Red 3 - 15 months
O-Benzyl-p-Chlorophenol - 13 weeks
O-Nitroanisole - 13 weeks
1-Amino-2,4-Dibromoanthraquinone - 9 months
C.I. Acid Red 114 - 9 months
C.I. Acid Red 114 - 15 months
C.I. Direct Blue - 15 months
Hydroquinone - 15 months
4-Hydroxyacetanilide - 15 months
1-Amino-2,4-Dibromoanthraquinone - 15 months
Pentachloroanisole - 15 months

TABLE 3
CHRONIC BIOASSAYS

Navy Fuel JP-5
Dimethyl Vinyl Chloride (mice)
Methylmethacrylate (mice & rats)
Tetrachloroethylene inhalation (mice)
Dichloromethane (mice & rats)
Phenylephrine HCl (mice & rats)
Dimethyl Methylphosphonate (mice & rats)
p-Dichlorobenzene (rats)
Erythromycin Stearate
Chlorpheniramine Maleate (mice & rats)
C.I. Acid Orange 3 (rats)
Oxytetracycline HCl (mice & rats)
Tetrakis(Hydroxymethyl)Phosphonium Chloride
(mice & rats)
Ethylene Oxide (mice)
Boric Acid
Methyl Carbamate (mice & rats)
Pentachloronitrobenzene (mice)
Bromodichloromethane (male mice & female rats)
N-Phenyl-B-Naphthylamine (mice)
Mixed Xylenes (mice)
Nitrofurantoin (mice)
Rotenone (mice & rats)
Rhodamine (rats)

TABLE 4
COMPLETED DATA AUDIT REVIEWS

<u>Chemical Name</u>	<u>Chemical Manager</u>	<u>Lab</u>	<u>Audit Team</u>
Chloramben	J.K. Dunnick	GSRI	NTP
Chlorobenzene	W.M. Kluwe	BCL	NTP
Dichloromethane (inhalation)	J.H. Mennear	BNW	NTP
Melamine	R.L. Melnick	LBI	NTP
o-Phenylphenol (mice)	M.I. Luster	BCL	NTP
4-vinylcyclohexene	J.J. Collins	LBI	NTP
Ampicillin Trihydrate	D.W. Bristol	Springborn	NTP
N-Butyl Chloride	J.H. Roycroft	Mason	NTP
Chlorendic Acid	J. French	HZ	NTP
Chlorowax 500c	J.R. Bucher	SoRI	NTP
Chlorowax 40	J.R. Bucher	SoRI	NTP
Decabromodiphenyl Oxide	H.B. Matthews	HZ	NTP
1,2-Dichloropropane	J.C. Lamb	Mason	NTP
Diesel Fuel Marine	M.P. Dieter	LBI	NTP
Di(2-ethylhexyl)adipate	J.E. Huff	Mason	NTP
Dimethylvinyl Chloride	B.A. Schwetz	LBI	NTP
Ephedrine Sulfate	R.D. Irwin	PRL	NTP
Geranyl Acetate	K.M. Abdo	SoRI	NTP
Methyl Methacrylate	P.C. Chan	BNW	NTP
Mirex	J.E. Huff	FCRC	NTP
Navy Fuel JP-5	M.P. Dieter	LBI	NTP
2,4 & 2,6-Toluene Diisocyanate	M.P. Dieter	LBI	NTP
Vinylidene Chloride	R.S. Chhabra	GSRI	NTP

<u>Chemical Name</u>	<u>Chemical Manager</u>	<u>Lab</u>	<u>Audit Team</u>
Phenylephrine Hcl	J.R. Bucher	PRL	NTP
Chlorpheniramine Maleate	R.L. Melnick	BCL	NTP
1,4-Dichlorobenzene	J. Goldstein	BCL	NTP
Oxytetracycline Hcl	K.M. Abdo	PRL	NTP
Tetrakis(Hydroxymethyl) Phosphonium Chloride	C.W. Jameson	BCL	NTP
Tetrakis(Hydroxymethyl) Phosphonium Sulfate	C.W. Jameson	BCL	NTP
Nitrofurantoin	R. Kutzman	SoRI	NTP

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-ES-21068-01 CPB

PERIOD COVERED

October 31, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of Methyl Bromide in the Rat Forestomach

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: G. A. Boorman	Acting Chief	CPB	NIEHS
Others: H. L. Hong	Biologist	CPB	NIEHS
K. Yoshitomi	D.V.M., Ph.D.	CPB	NIEHS
C. B. Richter	D.V.M.	CMB	NIEHS
C. W. Jameson		CTEB	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Chemical Pathology Branch

SECTION

Tumor Pathology Section

INSTITUTE AND LOCATION

NIEHS, Research Triangle Park, N.C. 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Methyl bromide (MB) is administered by oral gavage in peanut oil to Wistar male rats five times weekly during 13, 17, 21 or 25 weeks. The animals are weighed and observed for clinical symptoms and sacrificed sequentially and examined to study the effects of MB in rat forestomach. This experiment is designed to determine if MB is carcinogenic to rats in 90 days.

Methods Employed:

Wistar male rats, 5 weeks weighing 80 to 100 g., were administered MB 50 mg/kg body wt. by gavage in 10 ml/kg body wt. of peanut oil 5 times a week during 13, 17, 21 or 25 weeks. Control rats received the equivalent of vehicle. MB solutions were prepared bi-weekly and analyzed both before and after the dosing to verify the concentrations. The animals were sacrificed sequentially to examine forestomach, esophagus, liver and lungs histopathologically. The stomach was opened along the greater curvature and stretched for fixation, after which two mediolongitudinal sections were made for microscopy.

Major Findings and Proposed Course:

During the study, the appearance and general behavior of the animals did not appear to be affected. It appears that the body weight gain of MB-treated rats is less than that of the control animals. Either ulceration or progressive lesions caused by MB in the forestomach of rat is to be investigated under different time course.

Significance to Biomedical Research and the program of the Institute:

The previous 90-day gavage study reported MB to be carcinogenic is open to question. A review of the slides from that study (Danse et al., TAP 72:262-271, 1984) did not reveal a single carcinoma according to NTP pathologists. This project is to either verify or refute the previous findings as well as to determine whether the forestomach lesions are progressive or will regress once the stimulus is removed.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-ES-21074-01 CPB

PERIOD COVERED

October 31, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of Glycol Ethers on Bone Marrow Parameters

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: H. L. Hong	Biologist	CPB	NIEHS
Others: G. A. Boorman	D.V.M., Ph.D.	CPB	NIEHS
J. Canipe	Biological Lab Tech.	CPB	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Chemical Pathology Branch

SECTION

Tumor Pathology Section

INSTITUTE AND LOCATION

NIEHS, Research Triangle Park, N.C. 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Ethylene glycol (EG), ethylene glycol monomethyl ether (EGMME) or other glycol ethers is administered to BCF mice 4 consecutive days by gavage. Animals are killed on days 1, 5 and 14 following the final treatment to study the histopathology and myelotoxicity of glycol ethers.

Method Employed:

Male and female BCF mice, 5-6 weeks weighing 20-25 g., are given EG, EGMME or other glycol ethers at doses of 50, 100, and 250 mg/kg/day for 4 consecutive days by gavage. Histology, hematology and bone marrow parameters are assayed. Furthermore, use EGMME in perinatal BCF mice to study immune function and myelotoxicity.

Major Findings and Proposed Course:

It has been reported that EG is a weak reproductive toxicant, but a potential teratogen and a strong nephrotoxin; EGMME may produce thymic atrophy and mild leukopenia. This project is to determine if myelotoxicity is an additional feature of glycol ethers toxicity.

Significance to Biomedical Research and the program of the Institute:

We utilize glycol ethers in the murine hematopoietic model to examine a new toxic parameter that occurs in adult and perinatal BCF mice. Furthermore, the recovery in the hemopoietic system of mice following glycol ethers exposure is to be investigated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-ES-21080-01 CPB

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nuclear Magnetic Resonance Imaging Facility

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Morrow B. Thompson	Veterinary Pathologist	CPB	NIEHS
Others: R. R. Maronpot	Veterinary Pathologist	CPB	NIEHS
G. A. Johnson	Radiologist	Dept. of Radiology	Duke Univ. Med. Center

COOPERATING UNITS (if any)

Duke University Medical Center

LAB/BRANCH

Chemical Pathology

SECTION

Experimental Pathology

INSTITUTE AND LOCATION

National Institute of Environmental Health Sciences, Research Triangle Pk., N.C.

TOTAL MAN-YEARS

1

PROFESSIONAL

1

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Within the last nine months, significant progress has been made in the refinement of software and hardware for the imaging of small laboratory animals (rats) at Duke University. At this time (6/12/85), multiple (8 to 16) images can be acquired from an animal during the time required to produce one image. These images, which can be spaced 1.2 to 1.6 mm apart, can be used to closely examine a region of the animal that is 2.0 to 2.5 cm thick. This technique will be used to produce serial images of the brains, livers, and spleens of normal and experimental animals.

Preliminary experiments have been conducted using the high field (1.5 Tesla), large-bore (1.0 meter) system. In one experiment, rats that had been fed a hepatocarcinogen for 18 months were imaged. Multiple, discrete hepatic neoplasms ranging from 2 to 12 mm in diameter were readily identified on the images. The images were correlated with gross and histologic sections of the animals. By using a multiple spin echo sequence and by calculating T2 relaxation times, the prolonged relaxation times of the neoplasms were demonstrated. Extracellular space for the neoplasms and for normal liver was calculated using a morphometric analyzer and was compared with the T2 relaxation times for the same lesions. There was a strong, direct relationship between the T2 relaxation time and the percent of extracellular space for the lesions. This may be related to an increase in water content in the neoplasms.

Preliminary imaging experiments have also been conducted on rats with mononuclear cell leukemia (enlarged spleen) and on rats to demonstrate normal anatomic structures in the brain. Currently, a study is underway to examine the ability of nuclear magnetic resonance (NMR) imaging to detect pituitary neoplasms in rats.

Future studies will involve detailed protocols to examine the essential changes in the livers of rats exposed to a carcinogen. The imaging will be conducted throughout the exposure period and will be used to examine the time to tumor formation, the biologic behavior, and characteristics of hepatic neoplasms and the possible repression of neoplasms. Also, studies will begin which will explore the use of NMR imaging as a means of evaluating hepatic function in normal and experimental animals. This will involve the use of a compound (tagged with a paramagnetic metal) the combination of which will be actively removed by hepatocytes from portal blood and excreted into the bile. Imaging experiment of the brain will also continue.

ARGONNE NATIONAL LABORATORY, ARGONNE, IL 60439
(222Y01-ES-20091)

TITLE: Refinement and Use of Peraino Rat Liver Tumor Model in Investigation of Hepatocarcinogenesis

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Carl Peraino, Ph.D.

PROJECT OFFICER (NIEHS): R. R. Maronpot, D.V.M., Head, Experimental Pathology
Chemical Pathology Branch, TRTP, NTP

DATE CONTRACT INITIATED: September 29, 1982

CURRENT ANNUAL LEVEL: \$366,989.00

PROJECT DESCRIPTION

OBJECTIVES: Refine the neonatal rat short-term in vivo liver tumor model described by Peraino (Carcinogenesis 1981:2,463) to investigate mechanism of carcinogenesis. Utilize the refined model to test selected chemicals as initiators and promoters of hepatocarcinogenesis. A major emphasis will be placed on detailed histochemical characterization of "preneoplastic" foci and on investigation of mechanisms of hepatocarcinogenesis.

METHODS EMPLOYED: Neonatal rats will be dosed with known initiators and promoters to determine optimal dose response and timing of chemical administration. Effects of standard versus purified rodent diet on the liver tumor response will be determined. Multiple interim sacrifices will permit examination of the development and progression of preneoplastic alterations in the liver. Seven histochemical stains will be used to characterize the phenotype of "preneoplastic" lesions.

MAJOR FINDINGS AND PROPOSED COURSE: Tentative conclusions available from analysis of liver foci data indicate that Fischer 344 rats are less sensitive than Sprague-Dawley rats in the neonatal liver tumor model. Furthermore, it is apparent that NIH-07 diet is associated with less GGT foci than the number seen with purified diet. As initiator (DEN) dose is increased, there is an increase in phenotype complexity of foci with a plateau of this response at a dose of 32 micrograms. In terms of focus size, the more phenotypically complex foci are larger as a function of initiator dose. In terms of liver tumor incidence, initial findings indicate a substantial dose response to promotion by phenobarbital in Sprague-Dawley rats.

Additional findings will be available at the end of Fy 1985. Testing of selected chemicals in the neonatal model should commence by the second quarter of FY 1986.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The information gained from this research contract will help in the interpretation of liver tumor responses observed in conventional two-year rodent carcinogenesis tests and should provide basic insight into mechanism of carcinogenesis. The most frequent carcinogenic effect observed in NCI/NTP two-year carcinogenesis studies is the liver tumor response.

LITTON BIONETICS, INC., KENSINGTON, MD 20895
(N01-ES-3-5023)

TITLE: Refinement and Use of a Short-term In Vivo Rat Liver Tumor Model in Investigation of Mechanisms of Carcinogenesis

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Michael R. Moore, Ph.D.

PROJECT OFFICER (NIEHS): R. R. Maronpot, D.V.M., Head, Experimental Pathology, Chemical Pathology Branch, TRTP, NTP

DATE CONTRACT INITIATED: March 15, 1983

CURRENT ANNUAL LEVEL: \$321,369.00

PROJECT DESCRIPTION

OBJECTIVES: Refine a short-term in vivo rat liver tumor model such as that described by Pitot (Nature 1978:27,456) and test selected chemicals using the refined model. A major thrust of this effort is to address practical approaches that would lead to development of a protocol which could be used in laboratories without specialized expertise. Consequently, emphasis has been placed on short experiments without partial hepatectomy (PH) and on utilization of a single histochemical marker (gamma glutamyl transpeptidase or GGT) on paraffin sections.

METHODS EMPLOYED: Male and female rats will be administered known initiators and promoters with and without PH to determine optimal timing of chemical administration. Effects of standard rodent diet versus purified rodent diet, route of chemical administration, and sex on subsequent development of GGT+ foci and liver tumors will be determined. Once the model has been refined, chemicals which have previously been tested in conventional two-year carcinogenesis tests will be tested as initiators, promoters, and complete carcinogens in the short-term model.

MAJOR FINDINGS AND PROPOSED COURSE: Utilizing alcohol-fixed livers for GGT staining, it has been found that rats on standard NIH-07 diet have diffuse background GGT staining that limits ability to detect small GGT+ foci. This background staining is not present when rats are fed purified diet. It has been tentatively concluded that PH enhanced initiation (number of GGT+ foci) by DEN, AAF, and AFB1 in the Fischer 344 rat. It is apparent that 36 weeks is insufficient time for the development of grossly visible tumors in Fischer rats initiated with DEN, AAF, or AFB1. The altered focus response in rats initiated at 7 or 21 days of age without PH is equivalent to the response in rats initiated at 56 days with PH. Data from studies that have completed in-life phases are being evaluated.

Additional findings will be available at the end of FY 1985. Current plans are to commence using the PH model to test chemicals during FY 1986.

BOARD OF REGENTS OF THE UNIVERSITY OF WISCONSIN SYSTEM, MADISON, WI 53706
(N01-ES-3-5024)

TITLE: Refinement and Use of a Short-term In Vivo Rat Liver Model in
Investigation of Mechanisms of Carcinogenesis

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Henry C. Pitot, M.D., Ph.D.

PROJECT OFFICER (NIEHS): R. R. Maronpot, D.V.M., Head, Experimental Pathology,
Chemical Pathology Branch, TRTP, NTP

DATE CONTRACT INITIATED: March 15, 1983

CURRENT ANNUAL LEVEL: \$163,315.00

PROJECT DESCRIPTION

OBJECTIVES: Refine a short-term in vivo rat liver tumor model such as that described by Pitot (Nature 1978:27,456) and test selected chemicals using the refined model. A major thrust of this effort is to test a variety of initiators and promoters in the partial hepatectomy (PH) model and to define appropriate uses and limitations of stereologic measurements of "preneoplastic" lesions.

METHODS EMPLOYED: Effect of diet, age at initiation, delayed promotion, and sex on tumor response in the PH model will be determined in addition to dose responses for different liver tumor initiators and promoters. Early indicators of response will be assessed in addition to an actual tumor endpoint. Once the model has been refined, chemicals which have previously been tested in the conventional two-year rodent carcinogenesis tests will be tested as initiators, promoters, and complete carcinogens in the short-term model.

MAJOR FINDINGS AND PROPOSED COURSE: Successful transplantation of isolated GGT positive cells has been established. A computer program for quantitating multiple phenotypes of altered foci without an intermediate photographic image has been developed and is currently being used to evaluate study data. Several studies have been completed through the in-life phases and are now ready for evaluation. Tentative conclusions to date indicate that the Fischer 344 rat is less sensitive in this model than anticipated and that use of NIH-07 diet is less satisfactory for identification of GGT foci than is purified diet. Use of more than one histochemical marker for foci is indicated. The standard PH model works with initiators and promoters other than DEN and phenobarbital, respectively. Other initiators tested include MNNG, thioacetamide, DMBA and L-ethionine. TCDD, mestranol, BHA, WY-14,643, and ciprofibrate have been tested as promoters. Three manuscripts have been submitted for publication.

Additional findings will be available at the end of FY 1985. Testing of selected chemicals in the PH model is anticipated to commence in September 1985.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The information gained from this research contract will help in the interpretation of liver tumor responses observed in conventional two-year rodent carcinogenesis tests and should provide basic insight into mechanism of carcinogenesis. The most frequent carcinogenic effect observed in NCI/NTP two-year carcinogenesis studies is the liver tumor response.

RESEARCH TRIANGLE INSTITUTE - Research Triangle Park, North Carolina
(NIH-N01-ES-38044)

TITLE: Genetic Monitoring of Inbred Rodents

CONTRACTOR'S PROJECT DIRECTOR: R. Wayne Hendren, Ph.D.

PROJECT OFFICER (NIEHS): Ghanta N. Rao, D.V.M., Ph.D., Expert
Laboratory Animal Management, CPB

DATE CONTRACT INITIATED: March 2, 1983

CURRENT ANNUAL LEVEL: \$66,837

PROJECT DESCRIPTION

OBJECTIVES:

The purpose of this contract is to provide genetic monitoring resource for biochemical genetic monitoring of each generation of inbred stock at all production facilities producing F344/N rats and B6C3F1 hybrid mice to the toxicology research and testing program of the NTP as well as the animals supplied to the testing facilities.

METHODS EMPLOYED:

Up to 15 designated loci will be monitored for each strain or hybrid by electrophoresis of erythrocyte lysates, kidney homogenates and serum proteins. Twenty parent generation animals of B6C3F1 hybrid mice or 10 hybrid mice and 5 F344 rats from each of the foundation colonies, production centers and one of the testing facilities will be evaluated for genetic integrity at monthly intervals. In addition frozen kidneys from 50 B6C3F1 hybrid mice per month received at the testing facilities for studies will be subjected to isoenzyme analysis by electrophoresis.

MAJOR FINDINGS AND PROPOSED COURSE:

Technical proficiency of the contractor in distinguishing the various allelic variants and hybrid types of rodent tissues has been evaluated. Parent generations of B6C3F1 hybrid mice, the hybrid mice, F344 rats and frozen tissues from the foundation colonies, production facilities, and testing laboratories are being evaluated for genetic homogeneity at monthly intervals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE

Fischer 344/N rats and B6C3F1 hybrid mice are the selected experimental animal species for evaluation of chemicals for toxicologic and carcinogenic properties under TRTP/NTP. The genetic integrity of the test animals is essential for developing reliable and accurate research data in animal experiments. Constant monitoring for biochemical genetic variants of rederived as well as production stock and test animals will ensure that data from NTP animal studies will be collected from genetically homogeneous rats and mice. Genetic homogeneity of test animals will be essential for program wide comparison of the background incidence of tumors and lesions between testing laboratories, between chemicals and within the same laboratory overtime.

SIMONSEN LABORATORIES - Gilroy, California
(NIH-N01-ES-45034)

TITLE: Rodent Foundation Colonies

CONTRACTOR'S PROJECT DIRECTOR: James D. Russell, Ph.D.

PROJECT OFFICER (NIEHS): Ghanta, N. Rao, D.V.M., Ph.D., Expert
Laboratory Animal Management, CPB

DATE CONTRACT INITIATED: April 1, 1984

CURRENT ANNUAL LEVEL: \$88,126

PROJECT DESCRIPTION

OBJECTIVES:

The purpose of this contract is to operate, maintain and provide pedigreed, defined microflora associated rodent foundation colonies for supplying breeders to the expansion colonies of the rodent production centers.

METHODS EMPLOYED:

Offspring from genetically defined inbred pedigreed stock will be Cesarian derived maintained in isolators and associated with defined microflora. Offspring from these foundation stock will be used to supply breeders to the expansion colonies of new rodent production colonies.

MAJOR FINDINGS AND PROPOSED COURSE:

Inbred pedigreed stock of Fischer 344/N rats and C57BL/6N and C3H/HeN MTV-mice from NIH repository were shipped to this contractor. Offspring from the mating of these pedigreed stock were Cesarian derived, maintained in isolators and associated with defined microflora. These foundation colonies are supplying 30 breeding pairs of Fischer 344/N rats and 45 breeding pairs of C57BL/6N and C3H/HeN MTV-mice per month to the expansion colonies of the TRTP/NTP rodent production centers.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE

Fischer 344/N rats and B6C3F1 hybrid mice are the selected experimental animal species for evaluation of chemicals for toxicologic and carcinogenic properties under TRTP/NTP. The genetic integrity of the parent generation of Fischer 344/N rats and the C57BL/6N and C3H/HeN MTV-mice to produce the B6C3F1 hybrid is essential for developing reliable and accurate research data in animal experiments. Centralized foundation colonies will provide better control on genetic integrity of all the breeders in the expansion and production colonies of rodent production centers.

EXPERIMENTAL PATHOLOGY LABORATORIES, INC., RESEARCH TRIANGLE PARK, N.C. 27709
(N01-ES-4-5035)

TITLE: Pathology Support for the NTP - Quality Assurance

CONTRACTOR'S PROJECT DIRECTOR: Dr. Jerry F. Hardisty

PROJECT OFFICER (NIEHS): Gary A. Boorman, D.V.M., Ph.D., Acting Chief,
Chemical Pathology Branch, TRTP, NTP

DATED CONTRACT INITIATED: June 30, 1984

CURRENT ANNUAL LEVEL: \$750,413.00

PROJECT DESCRIPTION

OBJECTIVES: The National Toxicology Program conducts short term and long term studies in rodents to determine the potential toxicity and carcinogenicity of chemicals found in the environment and workplace. Since the conclusions drawn from these studies have societal impact, it is crucial that the pathology data upon which the decisions are based are sound, accurate, and meet current standards. Therefore, the pathology data is subjected to a thorough pathology quality assessment. The objectives of this contract are (1) to provide pathology quality assurance review of all NTP studies, (2) provide pathology training to contract pathologists, and (3) photograph lesions for NTP pathologists and toxicologists who participate in pathology working groups, seminars, and workshops.

METHODS EMPLOYED: A team of histotechnicians and pathologists experienced in rodent pathology examine the data from each study. The histotechnicians examine each slide and verify the presence of the required tissues. In addition, a slide quality check is done. The pathologist reviews the pathology narrative, examines all target tissues (tissue in which a chemical effect is suspected), all tumors diagnosed and all tissues from 10% of the animals selected at random. All discrepancies between the original and QA pathologists are highlighted in a QA narrative submitted to the NTP. The discrepancies are reviewed by an NTP pathologist and selected cases are subjected to a pathology working Group (PWG) peer review. Approximately 30 two-year studies and 30 ninety-day studies are subjected to QA review each year (Tables 1 and 2). The QA pathologists also participate in NTP pathology roundtables, provide one-half day orientation sessions for contract pathologists, and assist in photography, description of lesions and review of lesions.

MAJOR FINDINGS: The chemicals subjected to QA were:

Dimethyl Methylphosphonate	Rats and mice
p-Dichlorobenzene	Rats
Mirex	Rats
(Methylene Chloride) Dichloromethane	Rats and mice
Oxytetracycline Hcl	Rats and mice
THPC	Rats and mice
Chlorpheniramine maleate	Rats and mice
Bromodichloromethane	Rats, female and male
Ethylene Oxide	Mice
Boric Acid	Mice
Pentachloronitrobenzene	Mice

Methyl Carbamate	Rats and mice
N-Phenyl-B-Naphthylamine	Mice
Mixed Xylenes	Mice
Nitrofurantoin	Mice
Rotenone	Rats and mice
Malonaldehyde (special review)	Rats and mice
Tetrachloroethylene (special review)	Rats and mice
Rhodamine 66	Rats
para-Chloroaniline	8 Mice
1,2-Epoxybutane	Rats and mice

PATHOLOGY ASSOCIATES, INC., IJAMSVILLE, MD. 21754
(N01-ES-45045)

TITLE: Pathology Support for the Toxicology Research and Testing Program

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Michael Stedham

PROJECT OFFICER (NIEHS): C. A. Montgomery, Jr., D.V.M., Head
Toxicologic Pathology, Chemical Pathology Branch

DATE CONTRACT INITIATED: September 28, 1984

CURRENT ANNUAL LEVEL: \$508,960.00

PROJECT DESCRIPTION

OBJECTIVES: To provide (1) the necessary personnel and resources to evaluate pathology materials, (2) histology, necropsy, and histopathologic interpretation, (3) personnel and resources for chairing or participating in NTP Pathology Working Groups, (4) Gross and/or microscopic photography support, (5) support for special projects.

METHODS EMPLOYED: The above objectives are carried out by use of standard pathology methods and equipment.

MAJOR FINDINGS AND PROPOSED COURSE:

A. Chaired Pathology Working Groups for:

1. HCCPD - rat, subchronic
2. Pentachloronitrobenzene - mice, chronic
3. 2-Mercaptobenzimidazole, subchronic
4. Dimethylmethylphosphonate - rat, chronic
5. THPC - mice, chronic
6. Dimethylvinyl chloride - mice, chronic
7. Phenylephrine Hcl - mice and rats, chronic
8. Isobutyl Nitrite, subchronic
9. Methyl Bromide, subchronic

B. Participated as member on Pathology Working Groups for:

1. Furan, 9 and 15-month interim sacrifice
2. Oxytetracycline - rats, chronic
3. C.I. Acid Orange 3 - rat, chronic
4. Phenylephrine Methylene Chloride - rats and mice, chronic
5. HCCPD - mice, subchronic

C. Trimming and slide preparation was provided for:

1. Napthalene - mice, chronic
2. Methylethylisocyanate - mice and rats, subchronic
3. Amsonic Acid - rats and mice, chronic
4. Resorcinol - rats, chronic
5. Argonne liver contract, rodent, special stains
6. 2,6-Dichloro-p-phenylenediamine - rat, special study of pancreas
7. Glycol Ether study - mice

D. Photography support was provided for the following chemicals:

1. DMMP - rat, chronic
2. Phenylephrine - rat, chronic
3. Mercaptobenzimidazole - rat, subchronic

E. Histopathologic interpretations was provided for:

1. Benzofuran - mice, low dose, chronic read-down
2. Amsonic Acid - rats and mice, chronic
3. Naphthalene - mice, chronic
4. Resorcinol - rats and mice, chronic
5. Diesel fuel marine and JP5 - mice, special review of skin lesions
6. Special review of Klebsiella - associated glomerulonephritis in mice.
7. H.C. Red 3 - rats, chronic - review and graded nephropathy
8. Chlorowax 500 - rats, chronic - special review of kidney lesions and mononuclear leukemias
9. Chlorowax 400 - mice, chronic - special review of brain

F. Necropsy

1. Observed
 - a. H.C. Yellow 4 - rat, chronic
 - b. C.I. Acid Red 114 - rat, chronic
 - c. PPB mixture - rats, chronic
2. Performed
 - a. Carvone - rats, chronic

TACONIC FARMS, INCORPORATED
Germantown, New York 12526
(N01-ES-45053)

SIMONSEN LABORATORIES, INCORPORATED
Gilroy, California 95020
(N01-ES-45052)

TITLE: Rodent Production Colonies

CONTRACTORS' PRINCIPAL INVESTIGATORS: Mr. Richard G. Paganelli (N01-ES-45053)
Dr. James D. Russell (N01-ES-45052)

PROJECT OFFICER (NIEHS): Ghanta N. Rao, D.V.M., Ph.D., Expert
Laboratory Animal Management, CPB

DATE CONTRACTS INITIATED: September 30, 1984

CURRENT ANNUAL LEVEL: N01-ES-45053 = \$174,074
N01-ES-45052 = \$142,377

PROJECT DESCRIPTION

OBJECTIVES:

The purpose of these contracts is to establish new rodent production colonies and produce disease-free, genetically homogeneous and microbiologically defined F-344 rats and B6C3F1 hybrid mice for the NTP toxicology research and testing programs.

METHODS EMPLOYED:

Genetically defined inbred pedigreed animals associated with defined microflora are being supplied from TRTP/NTP rodent foundation colonies to establish pedigreed expansion colonies. Offspring from these pedigreed expansion colonies will be used to establish production colonies and to produce contractually specified number of each species at weekly intervals.

MAJOR FINDINGS AND PROPOSED COURSE:

The expansion colonies are being established to supply breeders to the new rodent production colonies. Animals from these new production colonies are expected to be available for chemical toxicology studies during the first quarter of FY '86. These new rodent production colonies will replace the rodent production colony contracts and agreements started in 1979.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The Toxicology Research and Testing Program (TRTP) under the National Toxicology Program (NTP) organizes and conducts a comprehensive interagency testing and research program focused on determining potential human health hazards due to exposure to chemicals. The toxicologic evaluation of chemicals is generally conducted through a sequence of experiments that involve acute, subacute, subchronic and chronic exposure of laboratory animals to chemical substances. The chronic rodent bioassay is the current preferred procedure for determining the carcinogenic potential of chemicals. Rats, mice and other small laboratory animals are appropriate species for evaluating toxicologic and carcinogenic properties of chemicals. Fischer 344/N rats and B6C3F1 hybrid mice are the selected experimental animal species for evaluation of chemicals for toxicologic and carcinogenic properties under NTP. At least two centralized production facilities for F344 rats and B6C3F1 hybrid mice with homogeneous genetic properties and defined microbial status will insure an adequate and continuous supply of defined quality animals for the Toxicology Research and Testing Program.

MICROBIOLOGICAL ASSOCIATES, INCORPORATED
Bethesda, Maryland 20016
(N01-ES-45056)

UNIVERSITY OF ALABAMA IN BIRMINGHAM
Birmingham, Alabama 35255
(N01-ES-45057)

TITLE: Rodent Disease Diagnostic Laboratories

CONTRACTORS' PRINCIPAL INVESTIGATORS: Dr. James R. Ganaway (N01-ES-45056)
Dr. J. Russell Lindsey (N01-ES-45057)

PROJECT OFFICER (NIEHS): Ghanta N. Rao, D.V.M., Ph.D., Expert
Laboratory Animal Management, CPB

DATE CONTRACTS INITIATED: September 28, 1984

CURRENT ANNUAL LEVEL: N01-ES-45056 = \$235,481
N01-ES-45057 = \$90,446

PROJECT DESCRIPTION

OBJECTIVES:

The purpose of these contracts is to provide rodent disease diagnostic laboratory support for monitoring the microbial status, health status and investigating any disease conditions of breeders and weanlings of TRTP/NTP rodent production colonies and the rodents on test in the toxicology testing laboratories.

METHODS EMPLOYED:

Retired breeders and weanlings from the production colonies are being shipped to the rodent disease diagnostic laboratories at approximately monthly intervals for evaluation of viral and mycoplasmal serology profiles, pathogenic microorganisms, ectoparasites and endoparasites. These evaluations include detailed macroscopic examination of organs by necropsy and histologic examination of selected tissues for microbial and parasitic lesions. Serum samples from the sentinel animals of the toxicology studies are being evaluated for viral and mycoplasmal antibody profiles. In addition, sentinel animals and tissues from animals on studies are being evaluated for any suspected microbial and parasitic disease conditions.

MAJOR FINDINGS AND PROPOSED COURSE:

Routine and frequent evaluation of the animals from the production colonies for microbial status and disease conditions is essential for production of disease free and defined microflora associated animals for toxicology studies. Evaluation of the sentinel animals of the toxicology studies and tissues of animals with suspected disease conditions is useful in the control of infections and elimination of diseases in the testing laboratories. These programs will be continued to insure the production of disease and infection free, defined microflora associated rats and mice for chemical testing and to understand and document the disease conditions and infections in the animals of the chemical toxicology studies.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The Toxicology Research and Testing Program (TRTP) under the National Toxicology Program (NTP) organizes and conducts a comprehensive interagency testing and research program focused on determining potential human health hazards due to exposure to chemicals. The toxicologic evaluation of chemicals is generally conducted through a sequence of experiments that involve acute, subacute, subchronic and chronic exposure of laboratory animals to chemical substances. The chronic rodent bioassay is the current preferred procedure for determining the carcinogenic potential of chemicals. Rats, mice and other small laboratory animals are appropriate species for evaluating toxicologic and carcinogenic properties of chemicals. Fischer 344/N rats and B6C3F1 hybrid mice are the selected experimental animal species for evaluation of chemicals for toxicologic and carcinogenic properties under NTP. Routine and periodic evaluation of the animals in the production colonies for microbial status and disease condition will help to ensure production of infection and disease free animals for toxicologic evaluation of chemicals. Evaluation of the sentinel animals and animals on test with suspected disease conditions for microbial status and disease related lesions will help to document the infections and disease conditions. These procedures will also aid in interpretation of chemical induced changes and lesions in tissues and organs.

PUBLICATIONS

Rao, G.N. et al, "Ankylosis of Hock Joints in Group Housed B6C3F1 Male Mice". Presented at the 35th Annual Meeting of the American Association for Laboratory Animal Science (1984).

Rao, G.N. et al, "Utero Ovarian Infection in Aged B6C3F1 Mice". To be presented at the 36th Annual Meeting of the American Association for Laboratory Animal Science (1985).

EXPERIMENTAL PATHOLOGY LABORATORIES, INC
(N01-ES-45065)

TITLE: Management of the NTP Pathology Archives

CONTRACTOR'S PROJECT DIRECTOR: Dr. Jerry F. Hardisty

PROJECT OFFICER (NIEHS): Gary A. Boorman, D.V.M., Ph.D., Acting Chief,
Chemical Pathology Branch, TRTP, NTP

DATE CONTRACT INITIATED: September 30, 1984

CURRENT ANNUAL LEVEL: \$813,342.00

OBJECTIVES: During the past 15 years, the National Cancer Institute, now the National Toxicology Program (NTP), has conducted over 300 two-year studies (often preceded by 90-day toxicology studies) in rats and mice. The slides, blocks, and wet tissues from these studies are archived for use by the NTP and other interested parties. The NTP Archive contract objectives are to maintain this unique national resource in a dynamic "user friendly" basis. In addition, all new studies are inventoried, slide/block match-up performed, wet tissues checked for correct label, presence of untrimmed lesions and presence of correct animal identification. The NTP Archive personnel provide answers to questions that arise during the preparation of technical reports. They provide black and white plus color photographs of selected lesions for NTP pathologists and chemical managers. The Archive personnel trim in lesions uncovered during audits, provide pathology data coordination, and schedules Pathology Working Groups (PWG).

METHODS EMPLOYED: The above objectives are carried out by pathologists, histotechnologists and data clerks using standard methods.

MAJOR FINDINGS AND PROPOSED COURSE:

- A. Histology support was provided for untrimmed lesions found during audits.
- B. Pathologists verified the presence of untrimmed lesions and diagnosed lesions that were trimmed in.
- C. Gross photographs and microscopic photographs of lesions from over 20 studies were prepared for NTP personnel.
- D. QA pathologists participated in over 15 pathology working groups.
- E. Since January, 1985 - Provide 1-2 data clerks for each PWG to red-line pathology tables and check pathology data changes.
- F. Participate in the NTP conference on brain tumors in man and animals.
- G. Conduct orientation sessions for NTP contract pathologists.
- H. Stage mononuclear cell leukemia for several recent NTP studies prior to the preparation of the technical report.

I. The NTP Pathology Coordinator performed the following tasks:

- 1) Updated and maintained PWG schedule.
- 2) Distributed materials and slides following PWG review.
- 3) Updated and maintained the Tumor Pathology Section chronic bioassay schedule.
- 4) Maintained a centralized listing of NTP/BOA chemicals on chronic and subchronic tests at subcontractor laboratories.
- 5) Maintained a master schedule for monitoring the progress of chemicals on chronic bioassay tests.
- 6) Maintained Compound Information Summary Forms for subchronic toxicity test submitted to the Toxicologic Pathology Section.
- 7) Maintained schedule of interim kill chemicals.
- 8) Prepared compound status summary report for all chemicals to be terminated in FY 1984.
- 9) Prepared preminutes for Testing Status meeting.
- 10) Received and distributed Early Death Individual Animal Data Records.
- 11) Prepared an address list of chemical managers, project officers, and principal investigators for each contractor laboratory and chemical.
- 12) TDMS supported studies to data review - includes PEIS forms.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The NTP Archive is a unique national resource of over 5.5 million histological slides of spontaneous and induced lesions in rats and mice. The data is maintained in an access limited protected environment and is computerized for easy data access. This resource can be used for retrospective studies, to verify completed studies and to access and review new studies in an orderly manner. Interested parties can review pathology data from studies after receiving permission from the chief of the Chemical Pathology Branch.

UNIVERSITY OF MISSOURI, COLUMBIA, MO 65201
(NIH-1-U01-E503685-01)

TITLE: Cholecystokinin in corn oil induced pancreatic growth

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Travis E. Solomon

PROJECT OFFICER (NIEHS): Gary A. Boorman, D.V.M., Ph.D., Acting Chief
Chemical Pathology Branch

DATE CONTRACT INITIATED: September 28, 1984

CURRENT ANNUAL LEVEL: \$95,836.00

PROJECT DESCRIPTION

OBJECTIVES: To determine the role of pancreatic trophic hormones in vegetable oil induced pancreatic proliferations.

METHODS EMPLOYED: F344 rats will be given various levels of vegetable oils by gavage with and without administration of pancreatic trophic peptides. Pancreatic growth, pancreatic secretory response and regulatory peptide release will be determined by measuring pancreatic weight, pancreatic juice secretion, and blood hormone levels using radioimmunoassay.

MAJOR FINDINGS AND PROPOSED COURSE: Those studies were initiated in FY 84 and focused on method development. It has been possible to administer peptides both subcutaneously in gelatin and intravenously.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These studies should provide useful information about the potential role of cholecystokinin, secretin and neurotensin as mediators of corn oil induced pancreatic growth.

DARTMOUTH MEDICAL SCHOOL, HANOVER, N.H. 03755
(NIH-1-U01-E503687-01)

TITLE: Effect of fat intake on growth of pancreatic nodules

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Daniel S. Longnecker, M.D.

PROJECT OFFICER (NIEHS): Gary A. Boorman, D.V.M., Ph.D., Acting Chief,
Chemical Pathology Branch, TRTP

DATE CONTRACT INITIATED: September 28, 1984

CURRENT ANNUAL LEVEL: \$166,012.00

PROJECT DESCRIPTION

OBJECTIVES: The objectives are to evaluate the significance of the increased incidence of proliferative acinar lesions and their relationship to dietary oil. Specifically, gavage oil versus dietary oil (bolus effect), Lewis rat versus F344 rat response, growth potential of induced pancreatic lesions (cell culture and transplantation), presence of cellular oncogenes and role of dietary oil as a promotor or initiator of pancreatic lesions will be evaluated.

METHODS EMPLOYED: The studies employ gavage and feeding studies in rats utilizing routine pathology techniques plus cell culture and transplantation procedures.

MAJOR FINDINGS: During the first fiscal year of this cooperative, it was found that both Lewis rats and F344 rats respond to corn oil but that sensitivity of the strains vary. Studies in progress evaluate the effect of gavage versus dietary oil.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These studies will have relevance to the NTP in evaluating chemical carcinogenicity studies in which corn oil was the vehicle. In addition, those studies will be relevant to investigators involved in the role of nutrition and cancer.

NORTHWEST MEDICAL SCHOOL, EVANSTON, ILL
(NIH-1-U01-E503690-01)

TITLE: Vegetable Oils and Exocrine Acinar Cell Lesions

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. S. M. Rao

PROJECT OFFICER (NIEHS): Gary A. Boorman, D.V.M., Ph.D., Acting Chief,
Chemical Pathology Branch

DATE CONTRACT INITIATED: SEPTEMBER 28, 1984

CURRENT ANNUAL LEVEL: \$102,088.00

PROJECT DESCRIPTION

OBJECTIVES:

(1) To study the effect of short-term and long-term administration of vegetable oils on the DNA synthesis and mitosis of acinar cells and on the induction of proliferative acinar cell lesions, (2) to investigate the promoting effect of vegetable oils on 4-hydroxyquinoline and ciprofibrate initiated pancreatic lesions, and (3) to elucidate the biological properties of the acinar cell lesions induced by long-term administration of vegetable oils.

METHODS EMPLOYED: Male F344 rats will be fed semipurified diets containing various oils with or without prior administration of a pancreatic initiator and DNA synthesis and presence of lesions will be measured using standard techniques.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These studies will aid in the understanding of the initiation and promotion of pancreatic cancer which is a leading cause of death in man. These studies will also have relevance to rodent nutrition and toxicology studies where oil is used as a vehicle.

PATHCO, INC., POTOMAC, MD 20854
(N01-ES-55071)

TITLE: Pathology Support for the Toxicology Research and Testing Program

CONTRACTOR'S PRINCIPAL INVESTIGATOR: Dr. Paul K. Hildebrandt

PROJECT OFFICER (NIEHS): C. A. Montgomery, Jr., D.V.M., Head
Toxicologic Pathology, Chemical Pathology Branch

DATE CONTRACT INITIATED: January 4, 1985

CURRENT ANNUAL LEVEL: \$550,202.00

PROJECT DESCRIPTION

OBJECTIVES: To provide (1) the necessary personnel and resources to evaluate pathology materials, (2) histology, necropsy and histopathologic interpretation, (3) personnel and resources for chairing or participating in NTP Pathology Working Groups, (4) gross and/or microscopic photography support, (5) support for special projects.

METHODS EMPLOYED: The above objectives are carried out by use of standard pathology methods and equipment.

MAJOR FINDINGS AND PROPOSED COURSE:

A. Chaired Pathology Working Groups for:

1. Chlorpheniramine - rats, chronic
2. Acid Orange 3 - rats, chronic
3. Erythromycin - mice, chronic
4. Oxytetracycline - mice, chronic
5. Tripeleminamine - rats and mice, subchronic
6. Boric Acid - mice, chronic
7. Methylphenidate - rats and mice, subchronic
8. Furan - rats, 9 and 15-month interim sacrifice
9. Pyrilamine - rats and mice, subchronic
10. Triprolidine - rats and mice, subchronic
11. C.I. Acid Red 114 - rats, interim sacrifice
12. Methyl Carbamate - mice, chronic
13. 4,4'-Thiobis (6-t-butyl-m-cresol), subchronic

B. Participated as member on Pathology Working Group for:

1. Vinylcyclohexene Diepoxide, subchronic
2. Oxytetracycline Hcl - rats, chronic

C. Trimming and slide preparation was provided for:

1. Mercuric Chloride - rats, chronic
2. Mercuric Chloride - mice, chronic
3. Psoralen - mice
4. Wollastonite - rats, chronic
5. 1,2-Dichloroethane - rats, special review of kidneys and liver

D. Photography support was provided for the following chemicals:

1. Furan, interim sacrifices
2. Triprolidine, subchronic
3. H.C. Blue 1, chronic
4. Tripelennamine, subchronic

E. Histopathologic interpretation was provided for:

1. Orthophenylphenol, chronic, special review of target organs
2. Disperse Blue 1, chronic, special review of target organ
3. Chlorendic Acid, chronic, special review of salivary gland tumors and all fibrous histiocytomas
4. Monochloroacetic Acid - rats
5. Monochloroacetic Acid - mice
6. Mercuric chloride - rats, chronic
7. Mercuric chloride - mice, chronic
8. 8-Methoxypsoralen - mice, chronic
9. 1,2 - dichloroethane, rats, special study of target organs

F. Necropsy

1. Observed
 - a. PBB mixture - mice, chronic
 - b. ADBAQ - rats, chronic
 - c. Pentachloroanisole - rats, chronic

G. Site Visits/Audits

1. Resorcinol, IRDC
2. Amsonic Acid, IRDC
3. Annual Program review, Bioassay Systems
4. Annual Program review, E.G. & G. Mason Research Institute

SIMONSEN LABORATORIES, INCORPORATED
Gilroy, California 95020
(NIH-N01-ES-95643)

NATIONAL CANCER INSTITUTE
Frederick, Maryland 21701

TITLE: Rodent Production Centers

CONTRACTORS' PRINCIPAL INVESTIGATORS: Dr. James D. Russell (NIH-N01-ES-95643)
Dr. Joseph G. Mayo (NCI)

PROJECT OFFICER (NIEHS): Ghanta N. Rao, D.V.M., Ph.D., Expert
Laboratory Animal Management, CPB

DATE CONTRACT INITIATED: September 30, 1979

CURRENT ANNUAL LEVEL: NIH-N01-ES-95643 = \$262,863
(NCI) \$348,000

PROJECT DESCRIPTION

OBJECTIVES:

The purpose of these contracts is to produce disease-free, genetically homogeneous and microbiologically defined F-344 rats and B6C3F1 hybrid mice for the NTP toxicology research and testing programs.

METHODS EMPLOYED:

Genetically defined inbred pedigree stock was obtained from the NIH repository and shipped to each breeding facility. Offspring from matings between these animals were Cesarean derived, maintained in isolators, and associated with defined microflora. These animals are being used as inbred foundation stock for each breeding facility. Offspring from the foundation stock are used to supply production colonies from which contractually specified number of each species are produced weekly. Rats and mice are being shipped to contract toxicology testing facilities one week after weaning. Pedigreed production breeder stocks are being replaced every 30 weeks from the foundation colonies maintained in isolators.

MAJOR FINDINGS AND PROPOSED COURSE:

The F-344 rat and the B6C3F1 hybrid mouse serve as the primary animal test system for the NTP toxicology research and testing programs. The animals produced for these production facilities continue to be free of infectious diseases. Genetic monitoring has become an important aspect of the surveillance program. Each generation of inbred stock at all production facilities is being monitored for genetic integrity as well as diseases. The production colony at the Simonsen Laboratories will be terminated in June 1985.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The Toxicology Research and Testing Program (TRTP) under the National Toxicology Program (NTP) organizes and conducts a comprehensive interagency testing and research program focused on determining potential human health hazards due to exposure to chemicals. The toxicologic evaluation of chemicals is generally conducted through a sequence of experiments that involve acute, subacute, subchronic and chronic exposure of laboratory animals to chemical substances. The chronic rodent bioassay is the current preferred procedure for determining the carcinogenic potential of chemicals. Rats, mice and other small laboratory animals are appropriate species for evaluating toxicologic and carcinogenic properties of chemicals. Fischer 344/N rats and B6C3F1 hybrid mice are the selected experimental animal species for evaluation of chemicals for toxicologic and carcinogenic properties under NTP. At least two centralized production facilities for F344 rats and B6C3F1 hybrid mice with homogeneous genetic properties and defined microbial status will insure an adequate and continuous supply of defined quality animals for the Toxicology Research and Testing Program.

CLEMENT ASSOCIATES, INC. - Arlington, Virginia 22209
(N01-CP-95646)

TITLE: Pathology Support for the Carcinogenesis Testing Program

CONTRACTOR'S PROJECT DIRECTOR: Dr. Dawn Goodman

PROJECT OFFICER (NIEHS): Dr. E.E. McConnell, Chief, Chemical Pathology Branch

DATE CONTRACT INITIATED: September 30, 1979

CURRENT ANNUAL LEVEL: \$473,176

PROJECT DESCRIPTION

OBJECTIVES: To provide the necessary professional support and technical personnel and facilities to process tissues for light and electron microscopy; (2) perform the gross and/or histopathologic evaluation on animal tissues generated within the National Toxicology Program (NTP); (3) participate in advisory groups, workshops, seminars and site visits; and (4) conduct specific pathology support projects as directed by the NTP such as preparation of study sets, investigation of problems related to NTP pathology activities and quality assurance of occasional chemicals.

METHODS EMPLOYED: The above methods are carried out by use of standard histopathologic methods and equipment. They include those items commonly found in histology and pathology laboratories.

MAJOR FINDINGS AND PROPOSED COURSE: The contractor has been working on the following tasks:

- A. Chairing of Pathology Working Group meetings.
- B. Preparation of microslide study sets.
- C. Preparation of journal articles.
- D. Histopathologic evaluation of oleic acid.
- E. Special staining of islet cell tumors.
- F. Participation in the NTP Pathology Roundtable Meeting.
- G. Scheduling and assigning of subchronic QA and PWG activities.
- H. Pathology support in conduct of data audits.
- I. Subchronic pathology quality assessment on methyl bromide.
- J. Mirex review of additional liver sections, response to post-PWG.
- K. Pathology support to NTP Repository.
- L. Review of livers and stomachs of mice from the benzyl acetate study.
- M. Target organ review of HC Red 3.

- N. Combination QA/PWG on Temple University subchronic study of four psoralen compounds.
- O. NTP Meeting and demonstration of the Toxicology Data Management System (TDMS).
- P. Special staining of islet cell tumors.
- Q. Technical expertise to special meeting on pancreatic lesions.
- R. Interlaboratory variability in tumor rates - Phase I and II.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This quality assurance insures that the pathology arising out of studies conducted under the auspices of the NTP are valid and will stand up to peer review.

This project was completed in FY 85.

SYSTEMIC TOXICOLOGY BRANCH

SYSTEMIC TOXICOLOGY BRANCH Summary Statement

Ge

Prediction of the potential for chemicals to adversely affect human health is best accomplished through extrapolation from toxicological data collected in laboratory animals. Programs within the Systemic Toxicology Branch (STB), in combination with those of other branches in the Toxicology Research and Testing Program (TRTP), are designed to collect data to help characterize the toxicological profile of chemicals and also to collect data which help improve the methods for toxicological evaluation as well as better understand the mechanisms of toxicity of selected chemicals.

The Systemic Toxicology Branch consists of five groups: Biochemical Toxicology, Chemical Disposition, Fertility and Reproduction, Immunotoxicology, and Inhalation Toxicology. Each section is summarized below; for more details and specific accomplishments, consult the individual presentations on the following pages.

Biochemical Toxicology: Structure-activity studies of chemicals are done to ascertain the mechanisms of action at the molecular and biochemical level. Major projects involve the identification and characterization of chemically-induced alterations in cytochrome P-450(s). These enzymes are responsible for metabolism of exogenous chemicals. Studies are in progress to examine changes in the genetic control of various subspecies of cytochrome P-450 in the rat after treatment with several different environmental chemicals.

Chemical Disposition: Studies of chemical disposition under the NTP are conducted through four contracts and an in-house program consisting of three senior scientists plus post doctoral, student and technical support. The immediate objective of chemical disposition studies is to provide information on absorption, distribution, metabolism and excretion of chemicals or chemical classes chosen for testing in the NTP Bioassay. This information is intended for use in design and interpretation of results of studies of toxicity and carcinogenicity of these chemicals. Long-range, but equally important, objectives of chemical disposition studies are to develop and publish data which will permit a better assessment of structure-activity relationship which influence chemical disposition or mechanisms of toxicity and provide basic information which will facilitate the extrapolation of laboratory data to man. Projects in Chemical Disposition include studies of metabolism and disposition of a variety of chemicals, investigations of mechanisms of toxicity and metabolism and studies of the effects of age, body composition and route of administration on chemical disposition. Metabolism and disposition studies have addressed the fate of both industrial and environmental chemicals as well as chemicals commonly used in consumer products. Studies of mechanisms of chemical toxicity have included metals, metal complexes, halogenated aromatics and acrylate monomers. Studies of the effects of age, body composition and route of administration on chemical disposition indicate that each of these factors can have very selective effects and that the importance of these effects vary with the chemical administered and the mechanisms necessary to the metabolism and clearance of the respective chemical.

Fertility and Reproduction: Studies were conducted in in-house laboratories to assess the effect of various chemicals on reproduction and fertility in males

and females. These studies included such known reproductive toxins as glycol ethers and phthalate esters. Through contract mechanisms, studies continue which are designed to assess methods to detect adverse effects of chemicals on reproductive function or capacity. These include a program to assess continuous breeding trials as a means of assessing the effect of chemicals on fertility as well as evaluations on animals in subchronic toxicity studies to assess sperm morphology and sperm counts as well as vaginal cytology in rats. Studies were also initiated to characterize toxicity in neonatal rodents resulting from exposure to chemicals excreted in milk.

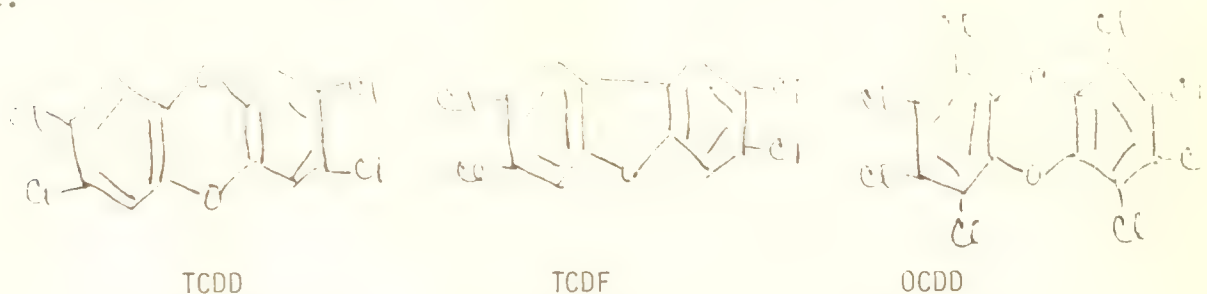
Immunotoxicology: Studies in this group continue to evaluate the influence of selected environmental chemicals on the immune system of animals, to relate alterations in immunological functions with both general toxicity and organ-specific toxicity, and to relate changes in immunological function with alterations in host resistance. This evaluation consists of a panel of immune and host resistance procedures which characterize immunotoxicity and correlate changes in immune function with altered host resistance. Data from these studies help characterize the toxicologic profile of chemicals, including those being evaluated for other toxicologic endpoints elsewhere in the NTP.

Inhalation Toxicology: The program of this group includes the design and execution of studies of compounds to which toxicologically significant exposure could be expected to be primarily by the inhalation route. Research is focused on manifestations of toxicity at the levels of tissues, organs, and organ systems. The in-house program is integrated with that of Northrop Services, Inc., an on-site contractor with responsibility for conducting research and testing by the inhalation route in an exposure facility within the in-house facility.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21003-05 STB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Disposition of Halogenated Dibenzofurans		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Linda S. Birnbaum Research Microbiologist	TRTP NIEHS
Others:	L.T. Burka Research Chemist	TRTP NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Systemic Toxicology Branch		
SECTION Chemical Disposition		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.2 0.5	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Halogenated dibenzofurans are found worldwide as environmental pollutants, Structurally related to other halogenated aromatic xenobiotics, their toxicity and disposition seem to vary with the degree and position of halogenation. This work has established that 2,3,7,8-tetrachlorodibenzofuran (TCDF), an extremely toxic isomer, is excreted only after metabolism and toxicity is inversely related to metabolic capability. The metabolites of TCDF produced by the rat are being characterized. There appear to be threshold body burden for toxicity. The distribution to the fetus was examined after maternal exposure. The role of body composition on the disposition of 2,3,7,8-tetrachlorodibenzodioxin (TCDD), the most toxic man-made compound known, is being examined in congenic mouse strains which are sensitive or resistant to TCDD toxicity. Absorption of octachlorodibenzo-p-dioxin (OCDD) from the gastrointestinal tract and the effect of dose on absorption is being studied in the rat.</p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: This work has used radioactively labeled compounds to quantify absorption, distribution, metabolism and excretion of TCDF, TCDD, and OCDD. TCDF was labeled with ^{14}C ; TCDD was labeled with ^3H or ^{14}C ; OCDD was labeled with ^{14}C . The disposition of TCDF has been studied after repeated exposure in guinea pigs. The disposition of TCDD has been studied in 4 strains of mice C57BL/6J Ah^b/Ah^b (responsive); C57BL/6J, Ah^d/Ah^d (non-responsive); DBA/J, Ah^b/Ah^b (responsive); DBA/2, Ah^d/Ah^d (non-responsive). The distribution to the fetus was determined by treating pregnant C57BL/6N mice during organogenesis and following the appearance and localization of ^{14}C TCDF and ^{14}C -TCDD in the fetuses. The effects of dose on the absorption of OCDD are being studied in the rat.



Analysis were facilitated by the use of a biological material's oxidizer and liquid scintillation counter. Metabolites are being purified and analyzed by thin layer chromatography and high pressure liquid chromatography. All data is subjected to further analysis by computer.

MAJOR FINDINGS AND PROPOSED COURSE:

Analysis of TCDF metabolites from rat bile has been initiated by preparing derivatives with trimethylsilane followed by purification by high pressure liquid chromatography. The derivatized metabolites will be analysed by GCMS.

C57BL/6J mice which are congenic at the Ah locus have been obtained from Dr. A. Poland (University of Wisconsin). They are being maintained by backcross/intercross techniques under a contract with RTI. These mice, which are genetically identical except at the Ah locus, where they are Ah^b/Ah^b (responsive) or Ah^d/Ah^d (nonresponsive), are now available for use to investigators at NIEHS. OCDD is a relatively abundant chlorinated dioxin pollutant. It is extremely insoluble and relatively non-toxic. In order to best determine doses for toxicity studies, the effect of dose on oral absorption and excretion is being studied. After iv exposure to $50 \mu\text{g } ^{14}\text{C}$ -OCDD/kg, less than 15% of the dose is excreted in the feces within 3 days. Most of the dose remains in the body, with the liver being the major depot, followed by skin and adipose tissue. However, after the same dose is given orally, more than 80% has been excreted with very little in the animal 3 days after treatment. As the oral dose is raised, more than 95% of it appears in the feces. Very low levels of radioactivity appear in urine. Approximately 1% of an iv dose appears in the bile within 5 hrs after treatment, but this is parent compound. Thus, OCDD seems to be poorly absorbed and metabolized.

The distribution of both TCDF and TCDD to the fetus was also examined using pregnant C57BL/6N mice, treated on day 11 of gestation and sacrificed from 24-96 hours later. In both cases doses were such that all the fetuses would have cleft palate but there would be no maternal toxicity. Even as early as one day after treatment, less than 0.5% of the TCDD dose and 0.05% of the TCDF dose were present in any embryo. This supports the potent teratogenic action of these compounds on the induction of cleft palate.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Halogenated aromatics are widespread and often highly toxic environmental pollutants. Compound and species related pharmacokinetic differences may help to explain variations in sensitivity to toxic effects.

PUBLICATIONS

McKinney, J.D., Chae, K., McConnell, E.E. and Birnbaum, L.S.: Structure-induction vs. structure-toxicity relationships for polychlorinated biphenyls and related aromatic hydrocarbons. Env. Health Perspect. In press.

Birnbaum, L.S.: The role of structure in the disposition of halogenated aromatic xenobiotics. Env. Health Perspect. In press.

Ioannou, Y.M., Birnbaum, L.S. and Matthews, H.B., Chronic toxicity and disposition of 2,3,7,8-tetrachlorodibenzofuran in male guinea pigs. J. Toxicol. Env. Health. 12:541-553, 1983.

Weber, H. and Birnbaum, L. S.: 2,3,7,8-Tetrachlorodibenzo-p-dioxin and 2,3,7,8-tetrachlorodibenzofuran in pregnant C57BL/6N mice: Distribution to the embryo and excretion. Arch. Toxicol. In press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21004-05 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Senescent Changes in Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Linda S. Birnbaum	Research Microbiologist	TRTP	NIEHS
Others:	William C. Eastin	Research Physiologist	TRTP	NIEHS
	Susan Borghoff	Graduate Student	TRTP	NIEHS
	Lililan Johnson	Biologist	TRTP	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

1.8

PROFESSIONAL

0.3

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Age-related changes in many physiological parameters have long been known to occur. The basis for these alterations is, however, not well understood. Response to various stresses seems to decline with age. Changes in the ability to metabolize exogenous as well as endogenous compounds has been suggested as a cause of altered functions. This work will explore senescent changes in metabolism of several tissues--liver, lung, kidney, small intestine, brain, lymphoid tissues. Enzyme systems such as glucuronyl transferase, B-glucuronidase, and those involved in intermediary metabolism and immune responses will be investigated. Altered distribution and excretion of chemicals in aging animals is being studied in order to elucidate the basis for age-related changes in toxicological responses. Age-related alterations in gastrointestinal absorption are also being studied.

PROJECT DESCRIPTION

This analysis of age-related changes in metabolism involves altered pharmacological properties related to the body's ability to handle various drugs and environmental chemicals and alterations in intermediary metabolism.

METHODS EMPLOYED: A colony of aging male Fischer F344 rats has been established by NIEHS at Charles River Laboratories. Weanling male rats, approximately 15 each month, are placed in the colony to be held until needed. An interim colony of retired breeder F344 male rats was maintained at NIEHS until rats reached 30 mo of age at Charles River. Animals available to us thus range in age from 1 through 36 months of age.

For studies of age-related changes, old animals (>24-27 mos) will be compared to young adult animals (2 mos) and to middle-aged ones (6-18 mos). These ages were picked to mimic major sacrifice and analysis times in the chronic toxicity tests. If necessary, additional ages will be used.

MAJOR FINDINGS AND PROPOSED COURSE: The disposition of several chemicals which are of interest to the NTP and which have been characterized in young rats will be studied in older animals. The absorption and excretion of compounds such as allyl isothiocyanate (AITC) (oil of mustard) and 4,4'-thiobis-6-t-butyl-m-cresol (TBBC) (a major rubber antioxidant) will be examined in old animals. These compounds are metabolized by different pathways and both qualitative and quantitative changes in metabolism will be assessed. The major metabolic pathway of AITC is through conjugation with glutathione and the formation of a mercapturic acid (MA). Urine is the major route of excretion, with approximately 75% of an oral dose being eliminated in the urine within the first 24 hrs. The metabolism and excretion of AITC was compared between male rats 3, 16 and 27 months of age. Urine, feces, CO₂ and volatiles were collected at various timepoints over a 72 hr. time period after treatment and total ¹⁴C-AITC-derived radioactivity was determined. Biliary excretion of AITC was also evaluated. Metabolic profiles were assessed in urine and bile samples. There was no age-related change in either the amount of radioactivity or in the metabolite profile that appeared in the urine. The amount of mercapturic acid eliminated was constant suggesting that older animals have the same ability to conjugate AITC with glutathione and form MA as do younger rats.

However, there were changes in the minor routes of AITC excretion. ¹⁴CO₂ expiration decreased in the senescent age group, coupled to an increase in exhaled volatiles. This implies that a decrease in the oxidation of AITC to CO₂ may result in a higher concentration of AITC in the blood available for exhalation. The percent of AITC-derived radioactivity in the feces decreased with age, but biliary excretion increased in middle age before declining in the oldest animals. Since more AITC-derived radioactivity appeared in bile than feces, it is likely that enterohepatic circulation of AITC is occurring and this may be changing with age.

TBBC is metabolized by conjugation with glucuronic acid. Animals 2.5, 16 and 26 months of age were treated with TBBC and the excretion in urine and feces

followed for 72 hrs. Biliary excretion was also evaluated. TBBC-derived radioactivity decreased with age in all excreta. HPLC analysis of bile did not show any qualitative change in the metabolite profile with age, but there was a quantitative change with age. This may be due to a decrease in glucuronyl transferase activity in the old animals. UDPGA levels were measured in kidney and liver samples from rats 2.5, 3, 6, 12, 20, 24, and 28 months of age. There was a decrease in the amount of UDPGA per gram of tissue with age. This suggests that the decreasing level of this cofactor may limit the conjugation of TBBC.

Age-related changes in absorption of xenobiotics from the small intestine are being investigated using the technique of in situ luminal perfusion. Initially, both passive and active transport was compared in young (2 mo) and old (27 mo) rats using model compounds. There was no change in passive absorption of 2-deoxy-d-glucose with age. Active transport of 3-O-methylglucose was half as rapid in old rats. This change occurred between 10 and 16 months of age. Ca absorption also decreased with age. The changes in this active transport system occurred between 3 and 12 months of age in both Fischer 344 and Long Evans rats. Chemicals of environmental interest will be used to see if age-related effects on the absorption of xenobiotics occur.

Age-related changes in intermediary metabolism were investigated in thymus, spleen, and macrophages from rats of 6, 12, 18, and 24 months of age. Pyruvate kinase and lactate dehydrogenase decreased in thymus, but increased in spleen. Glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase activities all decreased in pulmonary macrophages. These data suggest that the biochemical support for phagocytosis and cell-mediated immunity are diminished during aging in macrophages in the thymus, while the humoral immune response mediated by splenic B cells may not be compromised in senescent rats.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Changes that occur in toxic responses with advancing age have not been well documented. Altered responses to drugs have been known for some time, but the mechanism remains unclear. Studies of age-related changes in absorption, distribution, excretion and metabolism should better enable us to explain and predict how older organisms will respond to xenobiotic challenge.

PUBLICATIONS

Dieter, M., Wilson, R., and Birnbaum, L. S.: Age-related changes in glucose metabolizing enzymes in spleen, thymus, and pulmonary macrophages of F344 rats. Mech. Ageing Devpt. 26: 253-264, 1984.

Borghoff, S. J. and Birnbaum, L.S.: Age-related changes in glucuronidation and deglucuronidation in liver, small intestine, lung, and kidney of male Fischer rats. Drug Metab. Dispos. 13: 61-67, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21009-04 STB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Reproductive Effects in Males Exposed to Environmental Chemicals		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: James C. Lamb, IV	Research Biologist	STB NIEHS
Others: R.E. Chapin J.K. Dunnick	Staff Fellow Biologist	STB NIEHS CTEB NIEHS
COOPERATING UNITS (if any) Chemical Pathology Branch Data Management and Analysis Carcinogenesis and Toxicology Evaluation Branch		
LAB/BRANCH Systemic Toxicology Branch, TRTP		
SECTION Fertility and Reproduction Group		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 1.25	OTHER: 1.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Various environmental and industrial chemicals can disturb <u>male reproductive function</u>. The objective of these studies is to enhance our understanding of that toxic potential, and to elucidate the mechanism of action in chemicals found to be toxic. Chemicals which are active as chemosterilants in males, such as <u>glycol ethers</u>, <u>dimethyl methyl phosphonate</u>, <u>dibromochloropropane</u> and the <u>phthalate esters</u>, are used in various test systems. In addition to mechanistic studies, chemicals of unknown activity, such as the phenoxy herbicides and TCDD, have also been studied. Endpoints of toxicity include the assessment of <u>testicular morphology</u>, <u>spermatogenesis</u>, <u>sperm morphology</u>, and hormone levels. Studies continue on the morphological response of the testis to chemical exposure. <u>Androgen Binding Protein (ABP)</u> assays will also be performed to assess <u>Sertoli cell function</u>. Studies to evaluate early response of germ cells to toxicants are ongoing. Other studies are beginning to examine any changes of Sertoli cell function <u>in vitro</u> after exposure to toxicants <u>in vivo</u> and <u>in vitro</u>. Cell separation studies will examine biochemical defense mechanisms in isolated germ cell populations after exposure to various toxicants. A series of studies is beginning to evaluate the effects of acrylamide and its analogues on testicular and sperm functions. These studies are expected to yield valuable data on chemical toxicity in males, as well as improve the sensitivity and accuracy of future testing systems. </p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: In addition to histological evaluation of testes and accessory sex organs, these studies involve assessment of sperm head morphology from the cauda epididymis and vas deferens. The treated males have been studied by fertility and mating experiments and hormone patterns were studied in treated and control animals. Special high resolution light microscopic studies of the testis have been initiated. Testicular function will also be evaluated by measuring ABP and fluid secretion by the Sertoli cells. Fertility is evaluated by various mating trial protocols. Concurrent evaluation of numerous endpoints will let us identify the most sensitive, and the most cost-effective, endpoints to use in identifying compromised male reproductive function.

MAJOR FINDINGS AND PROPOSED COURSE: Studies have been conducted on the effects of DBCP, DMMP, glycol ethers and phthalate esters on the fertility of male rats. Subsequent studies are in progress which further investigate male germ cell toxicity as it related to fertility and testicular function using other model compounds which affect male reproductive function.

SIGNIFICANCE OF BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The important potential of chemicals to alter fertility and reproductive function is just beginning to receive appropriate attention. Studies are anticipated or are in progress using known mutagens and/or chemosterilants which will expand our knowledge of these chemicals' toxic mechanisms. Such information will allow us to develop more predictive test systems in this field.

PUBLICATIONS

Chapin, R.E. and Lamb, J.C., IV: Effects of ethylene glycol monomethyl ether on various parameters of testicular function in the F344 rat. Environ. Hlth. Perspect. 57: 219-224, 1984.

Chapin, R.E., Dutton, S.L., Ross, M.D., Sumrell, B.M., and Lamb, J.C., IV: Development of reproductive tract lesions in male F344 rats after treatment with dimethyl methylphosphonate. Exptl. Molec. Pathol. 41: 126-140, 1984.

Dunnick, J.K., Sollevelld, H.A., Harris, M.W., Chapin, J. and Lamb, J.C., IV: Dimethyl methylphosphonate induction of dominant lethal mutations in male mice. Mut. Res., 138: 213-218, 1984.

Chapin, R.E., Dutton, S.L., Ross, M.D., Sumrell, B.M., and Lamb, J.C., IV: The effects of ethylene glycol monoethyl ether on testicular histology in F344 rats. J. Androl. 5: 369-380, 1984.

Lamb, J.C., IV and Moore, J.A.: Effects of phenoxy acid herbicides and TCDD on male reproductive function. In Lobl, T.J. (Ed): Reproductive Health Care, pp. 269-286, 1984.

Lamb, J.C., IV and Chapin, R.E.: Experimental models of male reproductive toxicology. In Thomas, J.A., Korach, K.S., and McLachlan, J.A. (Eds.): Target Organ Toxicology: Toxicology of the Endocrine System, pp. 85-115, 1985.

Chapin, R.E., Ross, M.D., and Lamb, J.C., IV: Immersion fixation methods for glycol methacrylate-embedded testes. Toxicol. Pathol. 12: 221-227, 1984.

Chapin, R.E., Dutton, S.L., Ross, M.D., and Lamb, J.C., IV: Effects of ethylene glycol monomethyl ether on mating performances and epididymal sperm parameters in F344 rats. Fund. Appl. Toxicol. 5: 182-189, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21024-04 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Effects of Environmental Chemicals on Drug-Metabolizing Enzymes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Joyce A. Goldstein	Pharmacologist	TRTP	NIEHS
Others:	B. Furlong	Staff Fellow	TRTP	NIEHS
	P. Linko	Chemist	TRTP	NIEHS
	R. Weaver	Biological Lab Tech	TRTP	NIEHS
	P. McClellan-Green	Q-Appointment	TRTP	NIEHS
	H. Yeowell	Visiting Fellow	TRTP	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Biochemical Toxicology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

5.0

PROFESSIONAL

2.5

OTHER

2.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The objectives of this study are to examine changes in subspecies of cytochrome P-450 in the rat after treatment with environmental chemicals and to assess the implications of these changes.

1. Induction by a PCB Isomer: Dose response curves and time courses indicate that P-450c and P-450d are coordinately induced by 3,4,5,3',4',5'-HCB.
2. Methylenedioxyphenyl compounds (MDO) which act as suicide substrates induce different P-450 isozymes. They also differ in their ability to form complexes with different isozymes. Coadministration of methylcholanthrene with MDOs represses induction of P-450b by MDOs at the level of mRNA.
3. Structure-activity relationships of chlorobenzenes as inducers was studied. Hexachlorobenzene is the only 3-MC type inducer.
4. Two constitutive isozymes have been isolated P-450_H and P-450_G and antibodies raised to these forms.

The goal of this project is to better understand the changes occurring in metabolic capability of the liver toward chemical after exposure to environmental chemicals. Antibodies and cDNAs developed to these cytochromes will be useful in studying these effects.

PROJECT DESCRIPTION

MAJOR FINDINGS:

1. Three methylenedioxyphenyls were studied as inducers of P-450. Isosafrole (IS) induced three isozymes (P-450b, c and d) while t-butyl MDO induced only P-450b. Substitution of the methylene bridge decreased potency. The MDOs also differed in their ability to form metabolite complexes. Only IS formed complexes with d. 3-MC blocked P-450b induction at the mRNA indicating it represses this form.
2. Chlorobenzenes (CB) differed dramatically as inducers of MFOs. All induced phenobarbital forms (b+e), but only hexaCB induced the 3-MC forms (P-450 c and d). HexaCB also increased these mRNAs. It acted in 3-MC responsive strains of mice but not in nonresponsive strains. HexaCB had only minimal effects on binding of TCDD to the Ah receptor.
3. A constitutive isozyme, P-450_H, is decreased by 3,4,5,3',4',5'-hexachlorobiphenyl. P-450g is variable in the CD male rat on Western blots.
4. P-450_H metabolizes aflatoxin to mutagens at a relatively high rate. P-450g is less active. Neither metabolizes the premutagens or the flame retardant Tris-BP. We are also examining metabolism in hepatocytes.

PROPOSED COURSE:

We are studying the effects of sex, age, strain on constitutive P-450s. P-450g will be in the Fischer and CD rats. We are assessing the contribution of these constitutive forms to activation of mutagens in control liver. We are also examining the effects of chemicals on P-450b, c, and d, g and H and their mRNAs. Immunoassays are being developed for g and H. We are following the maintenance of P-450s in isolated hepatocytes and propose to examine the activation of the aminotoluenes (NTP chemicals) in isolated hepatocytes (mutagenesis, DNA repair, DNA binding).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The P-450 system is the principle monooxygenase system which catalyzes foreign chemicals as well as a number of endogenous compounds in genetic control of these enzymes. Probes developed in the rat will be particularly useful because of the wide use of this species in toxicology and carcinogenicity studies. These probes will also be useful for studying developmental and hormonal effects of regulation of this system. Probes may be used across species because of the similarity of many of these enzymes in various species.

PUBLICATIONS

Goldstein, J. A., Weaver, R. and Sundheimer, D. W.: Metabolism of 2-acetylaminofluorene by two 3-methyl cholanthrene-inducible forms of rat liver cytochrome P-450. Cancer Res. 44: 3766-3771, 1984.

Hardwick, J. P., Linko, P., and Goldstein, J. A.: Dose-response for induction of two 3-methylcholanthrene inducible forms of cytochrome P-450 in the rat and their mRNAs in rat liver indicating coordinate induction. Mol. Pharm., in press, 1985.

Goldstein, J. A.: Recent advances in elucidating the mechanism of induction of hepatic drug-metabolizing enzymes. Trends in Pharm. Sci., 5(7): 290-293, 1984.

Goldstein, J. A., Linko, P., Gasiewicz, T. A., and Yeowell, H.: Comparison of hexachlorobenzene with other chlorinated benzenes as inducers of hepatic cytochrome P-450 isozymes. Invited Chapter for International Symposium on Hexachlorobenzene (HCB) IARC, Lyon, France, June 24-28, 1985, in press.

Goldstein, J. A. and Hardwick, J.: Regulation of a multigene family of P-450 isozymes by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. Banbury Report 18: Biological Mechanisms of Dioxin Action: 119-133, Cold Spring Harbor Laboratory, 1985.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21026-04 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Disposition of Hexabromonaphthalene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Linda S. Birnbaum	Research Microbiologist	TRTP	NIEHS
Other:	James D. McKinney	Research Chemist	LEC	NIEHS
	Christopher Miller	Guest Worker	TRTP	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.2

PROFESSIONAL

0.2

OTHER

1.0

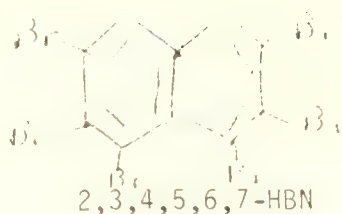
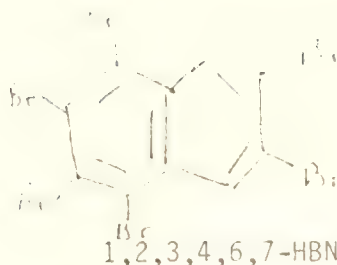
CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Bromonaphthalenes have no known industrial use or application, but have been identified as contaminants of Firemaster BP-6, the toxic mixture of polybrominated biphenyls used as a fire retardant and involved in a major episode of environmental poisoning in Michigan. Structurally related to other halogenated aromatic xenobiotics, their toxicity and disposition seem to vary with the position of bromination. This work has studied the chemical disposition of a mixture of 2 hexabromonaphthalenes (HBNs), previously identified as a single isomer, 1,2,3,4,6,7-HBN. The compound is incompletely absorbed after an oral dose. After iv treatment over 50% of the dose is excreted as metabolites within 3 days. However, the remainder of the dose seems to be extremely persistent, over 25% remaining in the liver after 35 days. These disposition results led to proof of the presence of two isomers by high resolution NMR, present in a ratio of 65:35 which have been identified as 1,2,3,4,6,7- and 2,3,4,5,6,7-HBN. The difference in the fate of the two isomeric has been proven by isolation and characterization by high resolution NMR of the HBN remaining in the liver 10 days after treatment. While the HBN dosed was in an isomeric ratio of 65:35 (1,2,3,4,6,7-:2,3,4,5,6,7-), the HBN in the liver 10 days after oral treatment was in the ratio of 20:80. The toxicity of this HBN mixture was examined in mice. A single oral dose as high as 1000 mg/kg had no toxic effects. However, repeat dose toxicity was detected at doses as low as 5 mg/kg for 7 days. The toxic response was toxicity similar to that seen for TCDD and related compounds. A complete teratology study was carried out and the teratogenic response was identical to that observed with TCDD, with the main endpoints being kidney anomalies and cleft palate at doses as low as 1 mg/kg.

PROJECT DESCRIPTION



METHODS EMPLOYED: This work has used ^{14}C -labeled compound to quantitate the chemical disposition of HBN in male Fischer 344 rats after acute oral and iv exposure. Analyses were facilitated by the use of a biological materials oxidizer and liquid scintillation counting. Tissue extraction with organic solvents was followed by thin layer chromatography in order to resolve parent compound from metabolites. Resolution of the compound into two isomers was accomplished using high resolution NMR. The extraction of non-radioactive HBN from liver was carried out by solvent extraction and chromatography. Teratological evaluation in C57BL/6N mice was carried out using standard procedures.

MAJOR FINDINGS AND PROPOSED COURSE: Recent studies from our laboratory had determined that HBN was a mixture of two closely-related isomers in an approximate ratio of 65:35. The major isomer was toxic, metabolized and cleared while the minor isomer was apparently non-toxic and very persistent. The major isomer was identified by high resolution NMR as 1,2,3,4,6,7-HBN. In order to conclusively identify the minor isomer and prove that it persisted, rats were treated with 2.5mg/kg HBN and held for 10 days. At that time, they were sacrificed, the livers were removed, extracted with chloroform/methanol. Separation of the halogenated aromatics from the lipids was achieved by chromatography on Sephadex LH-20. HBN was separated from fatty acids by acidic alumina chromatography. The purified HBN, which chromatographed with dioxins and furans, rather than PBBs, was then analyzed by NMR and found to contain two HBN isomers in the ratio of 20:80, confirming the earlier hypothesis that the minor isomer, which was now identified as 2,3,4,5,6,7-HBN, was persistent.

Because of the structural similarity of HBN to TCDD and other halogenated aromatic hydrocarbons and the similarity in toxic symptoms seen in the guinea pig with HBN and TCDD, we decided to investigate the teratogenic potential and potency of HBN. A large batch (~20g) was synthesized and characterized by NMR as having a ratio of 70:30 (1,2,3,4,6,7-HBN:2,3,4,5,6,7-HBN). Female C57BL/6N mice were treated with single oral doses to determine a toxic dose. No toxicity was detected with doses as high as 1000 mg/kg. However, treatment of pregnant dams on gestation days 10-13 with 10 mg/kg caused 100% induction of cleft palate. A complete teratogenic screen for both soft tissues and skeletal abnormalities after treatment on days 6-15 was performed. Maternal and fetal toxicity occurred at doses of 5 mg/kg. Hydronephrosis occurred at doses as low as 0.5 mg/kg. The threshold dose for cleft palate induction was 1 mg/kg and all the fetuses were affected at 2.5 mg/kg. Non-pregnant female mice treated with 5 mg/kg HBN for 7 days developed overt signs of TCDD-like toxicity. Thus, HBN appears to be a TCDD-like compound, but less potent than TCDD or TCDF.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It has been estimated that brominated naphthalenes may account for 20% of the toxicity of the Firemaster BP-6 mixture, and thus may be involved in the human and domestic animals toxicity observed in Michigan. The disposition of HBN relative to that of other halogenated aromatic compounds provides further understanding of the disposition of this broad class of compounds in the environment and better enables us to predict their risk to man.

PUBLICATIONS

Birnbaum, L. S. and McKinney, J. D.: A persistent hexabromonaphthalene isomer is 2,3,4,5,6,7-hexabromonaphthalene. J. Toxicol. Env. Health. In press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21031-01 STB

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Computer Simulation of Inhalation Exposures

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Michael P. Moorman

Engineering Officer

TRTP (NTP) NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Inhalation Toxicology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

.25

PROFESSIONAL

.25

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A computer simulation is being designed to model the distribution and disposition of compounds administered by inhalation. This will be used as a tool in the design of inhalation exposures and the interpretation of the resulting data.

PROJECT DESCRIPTION

OBJECTIVE: The objective of this project is to develop a computer simulation which models the distribution of compounds administered by inhalation to laboratory animals.

METHODS EMPLOYED: The computer simulation will be implemented using a version of 'SCOPE', a simulation environment developed by the National Biological Simulation Resource. The initial simulation will be based on published organ models and available physiological constants.

MAJOR FINDINGS AND PROPOSED COURSE: 1. This simulation will first be developed using simple models. Specific organ models will then be refined to better agree with experimental data. 2. This simulation can be used to estimate organ concentrations of compounds administered by inhalation and their metabolites as a function of time. This presents an investigator with the opportunity to test an exposure regime in an environment defined by explicit assumptions before actually performing the exposure. 3. Differences between estimated and actual results are used to evaluate basic assumptions and refine the organ models.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The use of computer simulations provides a practical approach to the difficult problem of studying the interactions of a compound with a complex environment. The judicious use of simulation will allow exposures to be designed more accurately, resulting in a savings of both time and resources.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21033-01 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Disposition of Xenobiotics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Linda S. Birnbaum Research Microbiologist TRTP NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

0.5

PROFESSIONAL

0.1

OTHER

0.4

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
(a1) Minors
(a2) Interviews
- ☐ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Pharmacokinetic factors can assist greatly in both dose-setting for toxicity studies and in interpretation of the results. Chemicals on-test by the NTP are nominated for disposition studies. The absorption (oral, dermal), distribution, metabolism, and excretion is studied in rats and other species as needed. The effect of dose is determined. In this way, the effects of chronic exposure may be predicted. The first chemicals to be studied in this project include o-benzyl-p-chlorophenol (BCP), and citral (oil of lemon).

PROJECT DESCRIPTION

METHODS EMPLOYED: Xenobiotics to be studied are radiolabeled with ^{14}C , or if necessary ^3H , by custom syntheses. Distribution and excretion are compared after iv, oral, and/or dermal exposure at several doses, the highest being 1/10th of the LD₅₀. Disposition after an iv dose is examined at varying time points after treatment. Excreta, expired air and volatiles are all analyzed for radioactivity which is resolved into parent compound and metabolites by organic solvent extraction and chromatography, TLC and/or HPLC. Metabolite characterization will be carried out by chemical and enzymatic means. Metabolite structures will be determined, if possible, by MS and NMR analyses.

MAJOR FINDINGS AND PROPOSED COURSE: BCP is a widely used disinfectant and germicide. It is highly irritating to the skin and causes renal tubular necrosis after subchronic exposures. Oral treatment results in an increase in liver weight and an induction of cytochrome P-450 b₅e, the forms also induced by phenobarbital. BCP binds to a constitutive form(s) of cytochrome-P-450 and inhibits arylhydrocarbon hydroxylase activity. BCP is well absorbed after oral exposure but at higher doses, absorption decreases. Part of a dermal dose remains at the site of application. BCP derived radioactively is excreted in approximately equal amounts in urine and feces. Urinary metabolites are glucuronide and glutathione conjugates. In the bile, glucuronide conjugates of BCP are the major metabolite although some glucuronides of hydroxylated-BCP are also produced. BCP metabolites tend to persist on the kidney and may play a role in the kidney toxicity observed after repeated exposure.

High environmental exposure occurs to citral (oil of lemon). This chemical, a mixture of two isomers, geraniol and nerol, occurs in detergents, cosmetics, and foods. Nothing is known about its disposition after dermal exposure. We intend to measure dermal absorption in male F344 rats and compare it to oral absorption and to disposition after iv treatment. Differential absorption and metabolism of the two isomers will be looked for. The effect of repeated exposure on disposition will also be studied.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Information on the absorption, distribution, metabolism, and excretion of chemicals nominated for toxicity testing by the NTP will improve our ability to set doses, interpret the results and extrapolate to the human situation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21034-01 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evolution of Metallothionein-like Proteins in Non-Mammalian Species

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B.A. Fowler Research Biologist STB NIEHS

COOPERATING UNITS (if any)

I. Armitage, Yale University; D. H. Petering, University of Wisconsin-Milwaukee;
C.F. Chignell and R. Hall; Laboratory of Molecular Biophysics; D. R. Winge,
University of Utah; J. S. Garvey, Syracuse University

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

1.0

PROFESSIONAL

0.5

OTHER

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The evolutionary patterns of the metallothionein family of proteins in nonmammalian species is an area of intense interest since these data may illuminate genetic mechanisms for the insertion of cysteine based sequences into this protein and the impact of this process on changes in structure and formation of the SH-mediated binding sites which characterize this protein. The American oyster (Crassostrea virginica) produces a low molecular weight cadmium-binding protein (CdBP) which is similar in size to metallothionein (MT) but which has a lower cysteine content and binds less Cd and no Zn. Studies of the CdBP binding site via kinetic analysis of the titration rate of SH groups by 5,5'-dithiobis-(2-nitrobenzoic) acid or 2,2 dithiobispyridine (DTP) showed a single phase reaction versus biphasis from MT. Circular dichroism studies of CdBP incubated in vitro with excess Cd disclosed a 40-50% reduction in the 259 nm Cd-S bond peak but no marked changes in the UV spectrum suggesting geometric alteration of the site. EDTA (1mM) chelation studies of CdBP showed that, like MT, release of Cd⁺² from the protein via this method was an extremely slow process which required days at 20°C as measured by the observed decrease in the 259 nm Cd-S circular dichroism peak. The above data indicate that the metal binding site of CdBP is similar to mammalian MT in that it is SH-mediated and resistant to EDTA chelation of Cd⁺² but also different with respect to the monopasic response to SH titration and unusual circular dichroic properties after addition of excess Cd⁺². Similar structural studies examining the nature of the SH-Mediated metal binding sites of the 45,000 dalton scallop kidney CdBP in relation to secondary structure are in progress. These data taken in concert with ongoing amino acid sequence and structural studies of oyster CdBP suggest that one evolutionary pathway for MT may involve the insertion of cysteine based sequences into a more ordered protein with concomitant changes in structure and metal-binding site formation or gene cleavage with production of a smaller more efficient molecule from a larger protein.

PROJECT DESCRIPTION

METHODS EMPLOYED: Purification of CdBP's from oyster and other marine invertebrates is being conducted following exposure to native Cd²⁺ or ¹¹³Cd and these proteins are isolated by standard column chromatography methods established in this laboratory. In addition, isolation of proteins by HPLC and gradient gel electrophoresis are also being conducted. Protein homogeneity is assessed by PAGE, SDS-PAGE, isoelectric focusing, amino acid analyses and amino acid sequencing. Biophysical studies will be conducted on these molecules as described above using circular dichroism, ¹H and ¹¹³Cd NMR, nanosecond fluorescence and ESR spin probe analysis of the metal-binding sites so that direct comparison with other known Cd-binding proteins may be made.

MAJOR FINDINGS: The previously reported low molecular weight cadmium-binding protein (CdBP) from the American oyster, *Crassostrea virginica*, has been further purified and characterized by improved technical methods. The internal organ distribution of the protein within the oyster and effects of life cycle/season on CdBP production also have been evaluated. CdBP isolated by extended ion exchange gradients or double ion exchange chromatography followed by HPLC analysis possesses an electrophoretic R_f of about 0.7 and contains relatively little Zn as previously reported. Cysteine, lysine and glycine are the dominant amino acids. When ion exchange columns are developed with NaCl gradients, the aromatic residues tryptophan, tyrosine and phenylalanine are found to be present, but these may be largely removed depending upon whether the protein is denatured and carboxyethylated prior to analysis. The ultraviolet absorption spectrum of CdBP also was variable with 250/280 nm ratios ranging from 17:1 immediately after ion-exchange chromatography to 2:1 following concentration procedures. Internal organ distribution studies showed that the visceral mass contained most of the Cd present with lesser amounts in the gills and mantle. In contrast with mammals, CdBP accounts for only about 30% of the total cell Cd burden in these tissues. Cu displacement of Cd from the protein is a particular problem during the summer spawning season and appears to stem from altered Cu metabolism during this period. Relative oyster dormancy during the winter also reduces CdBP production in response to Cd, and the protein is obtained most readily during the Fall and Spring. In summary, CdBP shares both similarities and differences with MT, and the magnitude of these parameters depends greatly on both the biology of the oyster and technical procedures used to isolate/characterize the protein.

Exposure of the scallop *Placopecten magellanicus* to 20ppb Cd²⁺ in seawater for 7 weeks results in a 7-fold increase in the kidney cytosol content of Cd and 5-fold increase in Zn. Sephadex G-75 column chromatography of the kidney cytosol showed that most of the Cd and Zn were bound to a protein complex with an estimated molecular mass of 45,000 daltons. Further purification of this complex by DEAE A-25 column chromatography disclosed the presence of 5 peaks with varying degrees of affinity for the ion exchange resin. One of these peaks (III) was successfully rechromatographed by ion exchange chromatography and further purified by HPLC using a gel permeation column. The resultant protein

peak which was resistant to disaggregation by 20 mM dithiothreitol gave a preliminary amino acid composition with cysteine, glycine, alanine and lysine as the major amino acids. The aromatic amino acid phenylalanine was also present. The ultraviolet absorption spectrum gave a 250/280 nm ratio of 2.5:1. Metal analysis of the purified protein showed it contained Cd, Zn and Cu in ratios of 1:1:1. Results of these studies indicate that scallop kidney produces a protein complex which appears to share both similarities with mammalian metallothionein with respect to the presence of both Cd and Zn but different with respect to apparent size, amino acid composition and ultraviolet absorption spectrum.

Proposed Course:

1. Complete biophysical studies on oyster CdBP which examine the relationship between amino acid sequence, structure and formation of SH-mediated binding sites in relation to other MT-like proteins.
2. Evaluate CdBP's in other species of molluscs, such as the scallop kidney which has a CdBP with an estimated molecular weight of approximately 45,000 to determine if the molecule(s) represent a dimer of a known MT-like protein or if protein chain-shortening has also occurred in the evolution of MT.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Metallothionein (MT) is a soluble metal-binding protein which has been extensively studied in mammalian systems where it appears to play a central role not only in zinc metabolism in normal animals but also in the intracellular binding and biological availability of Cd and Hg in animals exposed to these metals. This protein is characterized by a molecular mass of 6,800 daltons, an unusually high cysteine content (25-30%), an absence of aromatic amino acids and an extremely high metal-binding capacity (7 g atoms metal/mol protein).

Low molecular weight proteins, similar in size to metallothionein, have also been found in a variety of marine organisms. Some of these proteins appear to share both similarities and differences with MT. The relationships between differences in amino acid composition, metal-binding capacity and structure of these proteins are presently unknown but they are potentially of great significance to understanding the biological function of these molecules in vivo and determining possible evolutionary relationships to metallothioneins in more highly developed species.

PUBLICATIONS

Wood, J. M., Chakrabarty, A.M., Craig, P. J., Forstner, U., Fowler, B. A., Herms, U., Krull, I. S., Mackay, D., Olson, C. J., Russell, D. M., Solomons, W., and Silver, S.: Speciation in systems under stress. In Bernhard, M. and Brinckman, F. E. (eds): The importance of Chemical Speciation in Environmental Processes. Dahlem Workshop Reports. Springer-Verlag. (In press), 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 21036-03 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Disposition of Benzo(f)quinoline

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Linda S. Birnbaum
Lillian JohnsonResearch Microbiologist
BiologistTRTP NIEHS
TRTP NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0.3

PROFESSIONAL

0.1

OTHER

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Benzo(f)quinoline (BQ) has been recommended for study in the NTP as a mutagenic air pollutant and as a representative of nitrogen-containing aromatic heterocyclic compounds. It is present in various crude oils. Preliminary studies indicate that it may have carcinogenic potential. Before being tested in the bioassay program, disposition studies are needed to assess its absorption, distribution, metabolism and excretion. BQ was completely absorbed after oral exposure and was rapidly excreted as metabolites in approximately equal amounts into the urine and via the bile into the feces. No radioactivity persisted in the body after acute exposure. Such studies will not only result in more appropriate dose settings for toxicity studies, but a better understanding of the mechanism of toxicity of this compound.

PROJECT DESCRIPTION



METHODS EMPLOYED: ^{14}C -labelled benzo(f)quinoline (BQ) was used to study the disposition of this chemical in male Fischer 344 rats after acute oral and iv exposure. Analyses of radioactivity were facilitated by the use of a biological materials oxidizer and liquid scintillation counting. Tissue extraction with organic solvents was followed by high performance liquid chromatography in order to resolve parent compound from metabolites.

MAJOR FINDINGS AND PROPOSED COURSE: BQ was absorbed from the intestine, rapidly metabolized by the liver, and excreted in both urine and feces. Little remains in the body, since once metabolism occurs, the products are rapidly excreted.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Benzo(f)quinoline has been selected for carcinogenicity testing because of its mutagenic activity, its structural similarity to quinoline, a known carcinogen, and the potential for human exposure. No studies on its metabolism have been reported. These studies of its disposition will allow a better design to be developed for toxicity testing as well as provide more information on structure/activity relationships.

PUBLICATIONS

Birnbaum, L.S. and Johnson, L: Disposition of Benzo(f)quinoline in male rats. Drug Metab. Dispos. 13: 18-24, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21038-03 STB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chemical Metabolism and Disposition		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	H. B. Matthews J. M. Sanders	Research Chemist Biological Lab. Technician
		TRTP, NIEHS TRTP, NIEHS
COOPERATING UNITS (if any)		
LAB/BRANCH Systemic Toxicology Branch		
SECTION Chemical Disposition		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS 1.1	PROFESSIONAL 0.1	OTHER 1.0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Studies of <u>chemical metabolism</u> and <u>disposition</u> are designed to provide both <u>applied knowledge</u> in support of chronic toxicity tests conducted by the <u>National Toxicology Program</u> and <u>basic knowledge</u> of those <u>chemical structure</u> and <u>property relationships</u> which <u>determine toxicity</u> . Studies of methyl carbamate metabolism and disposition in rats and mice indicate that a species dependent variation in sensitivity to toxicity and carcinogenicity induced by this compound may be attributable to a much greater capacity of mice to metabolize and clear methyl carbamate. Studies of <u>ethyl carbamate</u> for structure activity relation to methyl carbamate indicate <u>structurally related variations</u> in toxicity may be attributable to subtle differences in kinetics of metabolism by mice.		

PROJECT DESCRIPTION

METHODS EMPLOYED: The absorption, distribution, metabolism and excretion of chemicals are studied following iv, oral or dermal administration of the chemical of interest to adult male rats and/or other species as the objectives of the studies dictate. Absorption and distribution of chemicals to tissues and clearance from tissues into excreta are quantitated by utilizing ^{14}C -labeled compounds serial sacrifice and analytical techniques which facilitate quantitation of radioactivity in biological media. Equipment used includes metabolism cages designed to permit separate collection of urine, feces and exhaled air, biological material oxidizers to convert organic compounds to CO_2 and liquid scintillation to quantitate ^{14}C in biological samples and CO_2 . Metabolite identification involves solvent extractions of tissues and excreta, purification by thin-layer and high pressure liquid chromatography and co-chromatography with authentic standards. Kinetic parameters are based on the disposition data and are calculated by computer.

MAJOR FINDINGS AND PROPOSED COURSE: Methyl Carbamate (MC), the subject of a recently completed bioassay, is widely used in textiles and is present in finished clothing. Preliminary results of a two-year study of toxicity and carcinogenicity indicate that MC may be carcinogenic to rats, and not carcinogenic to mice. The objective of the present study is to determine the nature of this species dependent toxicity. Results of these studies indicate that MC is readily absorbed from the gastrointestinal tract by both species and rapidly distributed to all tissues.

There is a marked difference in the manner at which rats and mice metabolize MC. Mice metabolize this compound much more rapidly than rats and accumulate much lower tissue levels with chronic exposure. It appears as if the species dependent toxicity of MC may be attributed at least in part to varying capacity to metabolize and discrete this compound.

Ethyl carbamate (EC) is an important industrial intermediate and a recognized carcinogen in the mouse. Studies of the metabolism and disposition of EC are underway in rats and mice to provide comparative data for structure/activity relation to MC which appears noncarcinogenic in mice. Results of these studies indicate that as with MC both species readily absorb and distribute EC and mice have a much greater capacity to metabolize and clear EC than do rats. However, the capacity of both species to metabolize and clear EC is dose dependent and easily saturated. The major metabolite is CO_2 and small amounts of polar metabolite is excreted in urine. Differences in the chronic toxicity and carcinogenicity of EC and MC may be attributable to subtle differences in the kinetics of in vivo metabolism.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROGRAM OF THE INSTITUTE: Chemicals vary widely in their properties and toxicities to higher animals. However, the health threat posed to humans by various chemicals cannot be accurately assessed prior to careful study. Therefore, the goal of laboratory research on chemical toxicity is to extrapolate those results to humans. Extrapolations of laboratory observations of chemical toxicities to humans are most relevant when based on

knowledge of chemical structure-activity relationships. Therefore, the goal of the present work is to gain a greater understanding of those chemical structure-activity relationships which impinge upon chemical metabolism, disposition, persistence and toxicity in higher animals. The significance of this work to biomedical research and the program of the Institute is that results of these studies help explain how various chemical toxicities are mediated and what steps can be taken to avoid or minimize chemical toxicities and thus provide a safer environment.

PUBLICATIONS

Matthews, H. B., Surles, J. R., Carver, J. G., and Anderson, M. W.: Halogenated biphenyl transport by blood components. Fund. Appl. Toxicol. 4:420-423, 1984.

Chopade, H. M. and Matthews, H. B.: Disposition and metabolism of p-nitroaniline in the male F-344 rat. Fund. Appl. Toxicol. 4:485-493, 1984.

Ioannou, T. M., Burka, L. T., Matthews, H. B.: Allyl isothiocyanate: Comparative disposition in rats and mice. Toxicol. Appl. Pharmacol. 75: 173-181, 1984.

Matthews, H. B.: Factors determining hexachlorobenzene (HCB) distribution and persistence in higher animals. Proceedings, International Symposium. Hexachlorobenzene (HCB) In Press, 1985.

Matthews, H. B., Chapade, H. M., Smith, R. W. and Burka, L. T.: Disposition of 2,4 dinitroaniline in the male rat. Xenobiotics (In press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 21044-01 STB

PERIOD COVERED

October 1, 1984 to September 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Dimethyl Hydrogen Phosphite: In vitro Metabolism by Rat and Mice Preparations

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Amin A. Nomeir

Senior Staff Fellow

TRTP, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0.6

PROFESSIONAL

0.5

OTHER

0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dimethyl hydrogen phosphite (DMHP) has been found to be a lung carcinogen in F344/N rats but not in B6C3F₁ mice. This study was designed to investigate the in vitro metabolism of DMHP by liver and lung preparations from rats and mice. The purpose of the study is to examine the role of metabolism in tissue and species selectivity to the toxic effects of DMHP. Preliminary results showed that DMHP is metabolized to formaldehyde by rat liver and lung microsomal mixed function oxidase systems. This metabolism was found to be NADPH dependent and the level of formaldehyde formation increased as the incubation time increased up to 2 hrs.

PROJECT DESCRIPTION

METHODS EMPLOYED: This work has utilized nonradioactive DMHP. Formaldehyde level was measured by Nash method. Liver and lung microsomal fractions were prepared by differential centrifugation. These fractions were washed by suspending in the same buffer and centrifugation. DMHP was incubated with the microsomal preparations in the presence and absence of NADPH at 37°C for various time intervals. Following incubation, the reaction was terminated and the level of formaldehyde was measured spectrophotometrically.

MAJOR FINDINGS AND PROPOSED COURSE: The major finding of this preliminary investigation is that DMHP is metabolized to formaldehyde by liver and lung microsomes in a time dependent manner. The extent of this metabolism will be examined under various conditions of substrate, NADPH, and protein concentrations using liver and lung microsomes from rats and mice. The effect of various factors on the formation of this metabolite *in vitro* will be investigated. Also the formation of formaldehyde by the microsomal fractions will be confirmed by other chemical and chromatographic methods.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: DMHP is used as an intermediate in the production of insecticides and herbicides, as an additive to lubricants and as a stabilizer in oil and plaster and was considered for use as a chemical to simulate the physical properties of anticholinesterase nerve gas agents; therefore, potential for human exposure in the production and use of this chemical is high. Since DMHP has been found to be a lung carcinogen to rats but not to mice, it is of interest to determine the nature of these species and tissue selectivity. The results of this study may offer a possible explanation as to the species and tissue specificity to DMHP toxicity and should prove useful to risk/benefit assessments regarding human health.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21046-02 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Postnatal Toxicology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator; (Name, title, laboratory, and institute affiliation)

PI: Lori A. Dostal Staff Fellow STB NIEHS

Others: B.A. Schwetz Supervisory Pharmacologist STB NIEHS
D.J. Kornbrust Senior Staff Fellow STB NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch, TRTP

SECTION

Fertility and Reproduction Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

2.0

1.0

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The objective of this study is to improve our ability to characterize the toxicity of drugs and chemicals to neonates relative to adults, and to explore the role of lactation in the induction of neonatal toxicity. Lactation is evaluated as a source of exposure to chemicals secreted into the milk and as the nutritional source for the newborn. A study was completed with DDE which established and validated various techniques, and generated information about the effects of this chemical on various parameters of lactation, including milk production, milk composition, pup growth, and mammary gland integrity. Another study was completed which characterized the secretion of the liver carcinogen, dimethyl-nitrosamine, into milk and the subsequent genetic toxicity in the suckling neonates. A new project was begun to determine the toxicity of di(2-ethylhexyl) phthalate (DEHP) to suckling rat pups of different ages. Toxicity was evaluated in the livers and testes of the suckling rats. A second new study was begun to quantitate the transfer of the histamine H₂-receptor antagonist, cimetidine, through the milk of lactating rats and to determine the antiandrogenic effects on the suckling male pups.

PROJECT DESCRIPTION

METHODS EMPLOYED: In order to obtain precise quantitative information about the disposition of a particular chemical or drug with respect to secretion into breast milk, samples of milk, blood and other fluids or tissues are collected at various times following administration of the substance to lactating experimental animals. Concentrations of the chemical and/or metabolites in the samples are determined by state-of-the-art analytical techniques. For the cimetidine study, an HPLC system was developed for the determination of the parent drug and its metabolites in rat milk and serum.

Gross toxic effects on the maternal animal or neonate are evaluated by monitoring body weight and clinical signs. The specific toxic effects observed after DEHP treatment include changes in liver, kidney and testis weights, increased hepatic palmitoyl CoA oxidase and carnitine acetyl transferase activities, and decreased plasma cholesterol and triglycerides. In the cimetidine study anogenital distance and serum testosterone are determined in the pups as an index of exposure to cimetidine in the milk.

MAJOR FINDINGS AND PROPOSED COURSE: A study in which the DDT metabolite, DDE, was administered daily (10 mg/kg body weight) to female rats beginning 5 weeks prior to mating and continued throughout gestation and the lactation period showed no toxicity to the dams and a slight increase in neonatal mortality in the DDE-treated group. The concentration of DDE in the milk of the treated rats was increased but there were no changes in any of the lactation parameters. When dimethylnitrosamine (DMN) was administered to lactating rats, the milk concentration of DMN was nearly identical to the plasma concentration of DMN at several different doses. The suckling neonates exposed to DMN via the milk showed increased DNA repair indicating that secretion of DMN into the milk may produce genotoxicity in neonatal rat liver.

The results of the studies of DEHP and cimetidine toxicity are not available as yet.

SIGNIFICANCE OF BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Information about the secretion of chemicals into milk and the resulting potential for neonatal exposure has been lacking, particularly for most industrial and many environmental chemicals. There is also very little information about the sensitivity of neonatal animals relative to adults to the toxic effects of many drugs and environmental contaminants. In addition, very few investigations of the ability of xenobiotics to adversely affect the neonate by impairing the maternal capacity for lactation have been performed. Thus, there is a considerable need to expand our knowledge in this area in order to permit reliable predictions about safe exposure levels for mothers with nursing infants. It is also anticipated that such efforts will stimulate refinements in existing methodology and establish novel techniques that will improve the quality of postnatal toxicology research.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 21055-01 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dimethyl Hydrogen Phosphite: Stability under Various Conditions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Amin A. Nomeir

Senior Staff Fellow

TRTP, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0.2

PROFESSIONAL

0.2

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dimethyl hydrogen phosphite (DMHP) has been found to be a lung carcinogen in F344/N rats but not in B6C3F₁ mice. It has been reported that DMHP is unstable in aqueous solution while it is stable as solution in methanol. No further information is available on the degree of unstability of this chemical in water or in any other organic solvent. This study was initiated in order to study the stability of this chemical in water, phosphate buffer pH 7.4 and some organic solvents. The purposes of these studies are a) to select an appropriate solvent to be used as a vehicle for metabolism studies; b) to get some understanding about the chemical stability of DMHP inside the animal; and c) to plan in vitro metabolism study using subcellular preparations from rats and mice.

PROJECT DESCRIPTION

METHODS EMPLOYED: An analytical method, using capillary gas chromatography has been developed to study the stability of DMHP. A Hewlett Packard Model 5880A gas chromatograph equipped with a split/splitless injector, flame ionization detector was employed. The column used was a 25 meters Db1 fused silica with an inside diameter of 0.25 M. The injector and detector temperature were kept at 150°C and 250°C respectively. The column temperature was programmed as follows: initial temperature at 40°C for 1 min then increased at a rate of 10°C/min to a final value of 150°C which maintained for 2 min. The retention time of DMHP averaged 3.75 min. Concentrations of DMHP were made at 100 mg/ml or 20 mg/ml in water, 0.1M phosphate pH 7.4, methanol or tetrahydrofuran and kept in a water bath at 37°C, room temperature 23°C, 8°C, -8°C or -70°C. At various time intervals, a volume was taken from this solution and diluted to an appropriate concentration (100ng/ul) for gas chromatographic analysis.

MAJOR FINDINGS AND PROPOSED COURSE: The major finding of this study, so far, is that DMHP is stable in water or buffer at 37°C for approximately 2-3 hrs then decomposed at a fast rate. The compound was also stable in water at room temperature for at least 3 hrs; however, at 24 hrs approximately 80% of the compound was decomposed. At 8°C DMHP in water was stable for 1 day then started to deteriorate, while at -8°C it was stable for 6 days then started to decompose. The rate of decomposition varied and was dependent on the storage temperature. The compound is stable as a solution in methanol for at least 4 weeks at room temperature.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: DMHP is used as an intermediate in the production of insecticides and herbicides, as an additive to lubricants and as a stabilizer in oil and plaster. The results of this study indicate that the compound is probably stable in the animal body for approximately 2-3 hrs then decomposes (chemically) to other chemical(s) (unknown at the present time). It also shows that in an *in vitro* metabolism study, the incubation time should not exceed 2 hrs at 37°C. Also it shows that in metabolism studies, water can be used as a vehicle for the compound as long as the solution is made fresh and stored under the appropriate conditions. The results of this study give us a better understanding of the stability of this compound and help in planning experiments and interpreting toxicological and metabolism results.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 21056-01 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dimethyl Phosphite: Comparative Metabolism and Disposition in Rats and Mice

PRINCIPAL INVESTIGATOR (If other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Amin A. Nomeir

Senior Staff Fellow

TRTP, NIEHS

COOPERATING UNITS (if any)

LABORATORY

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0.9

PROFESSIONAL

0.4

OTHER

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Dimethyl hydrogen phosphite (DMHP) has been recently tested by the National Toxicology Program for chronic toxicity and carcinogenicity in F344/N rats and B6C3F1 mice. DMHP was found to be carcinogenic to rat lungs but had no effect on mice. This study is designed to investigate various factors, related to pharmacokinetics, metabolism and disposition which may be involved in species sensitivity to DMHP.

PROJECT DESCRIPTION

METHODS EMPLOYED: This work will utilize [^{14}C]-labeled compound in order to study the absorption distribution, metabolism excretion and probably macromolecular binding of various doses of DMHP in F344/N rats and B6C3F₁ mice. Tissues from treated animals will be extracted and analyzed by high performance liquid chromatography to determine the parent compound and metabolites. Attempts will be made to identify various metabolites in tissues, expired air and excreta from both species.

MAJOR FINDINGS AND PROPOSED COURSE: Analytical methods using high performance liquid chromatography have been developed to examine the purity of the radioactive chemical. No major finding has been obtained from this study yet due to the lack of availability of [^{14}C] DMHP in a pure form. However, when we obtain the [^{14}C] chemical in a pure form the study will be initiated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: DMHP is used as an intermediate in the production of insecticides and herbicides and as an additive to lubricants. The potential for human exposure is high. Results from NTP study have shown that DMHP is lung carcinogenic to rats but not to mice. This study is designed to investigate the pharmacokinetics, metabolism and disposition of [^{14}C] DMHP in sensitive and insensitive species. The results of this investigation may offer a possible explanation as to the species selectivity to the carcinogenicity of DMHP. Knowledge of metabolism, pharmacokinetics and disposition by higher animals will also give a more accurate extrapolation to assess the toxic potential due to human exposure.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21057-01 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Pharmacokinetics and Metabolism of Neurotoxic Chemicals in Various Species

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Amin A. Nomeir

Senior Staff Fellow

TRTP, NIEHS

COOPERATING UNITS (if any)

Duke University Medical Center

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

0.8

PROFESSIONAL

0.2

OTHER

0.6

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type Do not exceed the space provided)

2,5-Hexanedione (2,5-HD) is believed to be the ultimate neurotoxic metabolite of the industrial neurotoxic solvents n-hexane and methyl-n-butyl ketone. Tri-o-cresyl phosphate (TOCP) is also a neurotoxic contaminant found in the commercial preparation tricresyl phosphate. These studies were initiated to investigate the absorption, distribution, excretion and metabolism of these neurotoxic chemicals in various species. It was also of interest to study the role of pharmacokinetics and metabolism in species sensitivity to neurotoxic agents. Analytical methods using capillary gas chromatography (GC) and high performance liquid chromatography (HPLC) were developed to analyze the parent compounds and their metabolites. Five metabolites of TOCP were synthesized and their structures were verified by various spectroscopic techniques. The metabolism of [¹⁴C]2,5-HD in the chicken was investigated following a dermal application of 50 mg/kg dose. The metabolism and disposition of TOCP was investigated following dermal application of [¹⁴C] labeled compound on the male cat. Also being investigated is the metabolism and disposition of orally administered TOCP to rats, cats and chickens.

PROJECT DESCRIPTION

METHODS EMPLOYED: Analytical methods using capillary GC and HPLC were developed to analyze n-hexane and its possible metabolites. A Varian Mode Vista 6000 GC equipped with a flame ionization detector and a 50 m glass capillary OV 101 column was used to analyze n-hexane and 11 of its possible metabolites. A Waters HPLC equipped with 5 μ M silica cartridge fitted into an RCM-100 radial compression separation system was used to analyze methyl-n-butyl ketone and 4 of its possible metabolites.

In the metabolism studies animals were treated with 50 mg/kg of the [^{14}C] labeled compound and the radioactivity in the excreta and exhaled air were collected and analyzed. Five groups of at least three animals were sacrificed at selected time points and the radioactivity was determined in the bile, tissues and the contents of the gastrointestinal tracts. Radioactivity was extracted from various tissues and excreta and analyzed by HPLC and GC to identify and quantify the parent compound and its metabolites.

MAJOR FINDINGS AND PROPOSED COURSE: 2,5-HD was absorbed from the skin of the chicken and distributed to all tissues examined and rapidly excreted as volatile organic materials (35.5%), $^{14}\text{CO}_2$ (11.9%) and in the urinary-fecal excreta (15%). Liver followed by the kidney and bone marrow contained the highest concentrations of radioactivity, while brain, spinal cord and sciatic nerve contained the lowest concentrations. The half-lives for the elimination of ^{14}C was longest for muscle (71 hr) and bone marrow (61 hr) and shortest for adipose tissue (12 hr). Most of the radioactivity in the plasma was identified as 5-hydroxy-2-hexanone followed by 2,5-HD and 2,5-dimethylfuran. These three chemicals disappeared biexponentially from the plasma with terminal half-lives of 7.6, 12.6 and 28 hr, respectively. 2,5-HD was the abundant chemical found in the liver, lungs and kidneys while 5-hydroxy-2-hexanone was the abundant chemical in the excreta.

The metabolism of a single dermal dose of 50 mg/kg of [^{14}C]TOCP was studied in male cats. TOCP was readily absorbed and subsequently distributed throughout the body. The total amount of radioactivity in the tissues reached its maximum level of 8.7% of the total dose at 24 hr following application and dropped slightly in the next four days. Generally, the highest concentrations of radioactivity were associated with bile, gall bladder, urinary bladder, kidneys, and liver while the lowest were found in the neural tissues, muscle, and spleen. Approximately 28% and 20% of the applied dose were recovered in the urine and feces, respectively within the ten day experimental period. TOCP was the predominant compound detected in the feces (26.3% of total fecal radioactivity) while a smaller percentage of the parent compound (2.3% of total urinary radioactivity) was detected in the urine. The major metabolite in the urine was *o*-cresol followed by di-*o*-cresyl hydrogen phosphate and *o*-cresyl dihydrogen phosphate; in the feces di-*o*-cresyl hydrogen phosphate was the predominant metabolite followed by *o*-cresol dihydrogen phosphate. Trace amounts of saligenin cyclic-*o*-tolyl phosphate, hydroxymethyl, and dihydroxymethyl-TOCP were also detected in the urine and feces. Other metabolites identified in the urine and feces were the stepwise oxidation products of

the methyl group of o-cresol. Unlike the feces, the bile contained mostly metabolites with trace amounts of TOCP detected at only 12 and 24 hr following application. o-Cresyl dihydrogen phosphate and di-o-cresyl hydrogen phosphate were the prevalent metabolites in the bile at all time points. Di-o-cresyl hydrogen phosphate and o-cresyl dihydrogen phosphate were the major metabolites in the plasma while dihydroxymethyl TOCP was present in trace amounts. An appreciable amount of saligenin cyclic-o-tolyl phosphate, which is believed to be the active neurotoxic metabolite, was detected in the plasma.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: 2,5-HD is believed to be the ultimate neurotoxic metabolite of the industrial neurotoxic solvents n-hexane and methyl-n-butyl ketone. These chemicals have been widely used in various industries, therefore potential for human exposure is high. Since these chemicals have been shown to cause neurotoxicity in humans as well as in laboratory animals, it was of interest to determine the fate of the active metabolite in the highly sensitive species (chicken) and the less sensitive species (rats). The objective of this work has been to determine which species, rat or chicken, serves as the best model for human exposure. It was also of interest to determine the pharmacokinetics and disposition of this neurotoxic metabolite in various species.

TOCP as well as many other organophosphorus compounds are known to cause a toxic condition described as organophosphorus induced delayed neurotoxicity (OPIDN). Some animal species (e.g., chickens, cats and humans) are sensitive while others (e.g. rats and mice) are not. This study was initiated to investigate the pharmacokinetics and metabolism of TOCP as a model compound for OPIDN in sensitive and insensitive species. This may give us a better understanding of the mechanism by which this group of organophosphorus compounds cause their effect and thus help in protecting man and his domestic animals from the toxic effect of these chemicals.

PUBLICATIONS

Nomeir, A. A. and Abou-Donia, M. B.: Analysis of n-hexane and related chemicals by capillary gas chromatography and high-performance liquid chromatography. Anal. Biochem. In press.

Nomeir, A. A. and Abou-Donia, M. B.: Studies on the metabolism of the neurotoxic tri-o-cresyl phosphate I - synthesis and identification by infrared, proton nuclear magnetic resonance and mass spectrometry of five of its metabolites. Toxicology. In press.

Nomeir, A. and Abou-Donia, M.B.: Studies on the metabolism of the neurotoxic tri-o-cresyl phosphate II - distribution, excretion and metabolism in male cats after a single dermal application. Toxicology. In press.

Abou-Donia, M. B. and Nomeir, A. A.: The role of pharmacokinetics and metabolism in species sensitivity to neurotoxic agents. Fundam. Appl. Toxicol. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 21059-01 STB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders) Gastric Toxicity of Acrylic Acid Esters		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	Burhan I. Ghanayem H. B. Matthews	Staff Fellow Research Chemist TRTP, NIEHS TRTP, NIEHS
COOPERATING UNITS (if any)		
Chemical Pathology Branch, TRTP, NIEHS		
LAB/BRANCH		
Systemic Toxicology Branch		
SECTION		
Chemical Disposition		
INSTITUTE AND LOCATION		
NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS 0.6	PROFESSIONAL 0.6	OTHER 0.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)		
<p> The <u>acute gastric toxicity of acrylic acid esters</u> was studied in F344 male rats. <u>Gavage administration of a single dose of ethyl acrylate (EtAc) or methyl acrylate (MeAc) caused dose- and time-dependent mucosal and submucosal edema and vacuolization of the tunica muscularis in the forestomach and submucosal edema in the glandular stomach. Equivalent sc or ip doses of EtAc did not produce similar gastric lesions. Structure-toxicity relationships revealed that MeAc was a more potent gastric toxin than EtAc, while acrylic acid and n-butyl acrylate were without effects. Gavage administration of equimolar doses of the saturated analogues of acrylic acid esters (methyl propionate or ethyl propionate) as well as methacrylic acid esters were without gastric toxicity. The gastric toxicity of acrylic acid esters was found to be dependent upon both acrylate ester concentration in dose vehicle and the lipophilicity of the dose vehicle (corn oil vs water). Gavage administration of 14 consecutive daily doses of EtAc produced no lesions in the glandular stomach, which indicates that prolonged insult with EtAc resulted in adaptation of the glandular stomach. On the other hand, similar treatment with EtAc caused a dose-dependent mucosal edema associated with vesicles, mucosal hyperplasia and hyperkeratosis, submucosal edema and inflammation, vacuolization of tunica muscularis and mucosal erosions or ulcers.</u> </p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: This work applied gravimetric techniques for the evaluation of gastric edema as well as histopathologic techniques for the evaluation and characterization of gastric edema and other gastric lesions.

MAJOR FINDINGS AND PROPOSED COURSE: Methyl acrylate and ethyl acrylate caused profound forestomach toxicity in F344 male rats. Structure-toxicity relationship studies revealed - 1) Methyl acrylate is more potent than EtAc, while acrylic acid and n-butyl acrylate were without effects, 2) The structural requirements for acrylic acid esters to cause gastric lesions include an intact ester molecule, a double bond, and no substitution at carbon number 2. Additional studies indicated that gastric toxicity may be attributed to the intact ester molecule or to metabolite(s) other than products of carboxylesterase-mediated hydrolysis (acrylic acid and alcohol) and that gastric toxicity is dependent upon both acrylate ester concentration in dose vehicle and the lipophilicity of the dose vehicle (corn oil vs. water). Glandular stomach lesions observed after 1, 2 or 4 daily gavage doses of 200 mg/kg of EtAc to F344 male rats include submucosal edema and inflammation. These lesions were not observed following 14 daily gavage doses. Therefore, it appears as if the glandular stomach became resistant to repeat EtAc gavage exposure with no apparent gastric alterations observed after 14 daily gavage exposures. On the other hand, forestomach lesions observed after 1, 2 or 4 daily gavage doses of 200 mg/kg of EtAc to F344 rats include mucosal edema and inflammation, vacuolization of tunica muscularis and mucosal erosions or ulcers. The forestomach adapted to the continuous insult by the development of a dose-dependent inflammation, hyperplasia and hyperkeratosis after 14 daily gavage doses of EtAc. The nature of this adaptation and the mechanisms involved in their induction by EtAc are currently being investigated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Acrylic acid esters are widely used in the production of polymers and copolymers for use in the preparation of latex paints, textiles, paper coating and specialty plastics. In a recent study conducted by the NTP, chronic administration of EtAc in corn oil by gavage resulted in a compound-related increase in the incidences of hyperkeratosis, inflammation and hyperplasia of the forestomach in the 13-week and 2-year studies. In the 2-year studies, EtAc caused squamous cell papillomas and squamous cell carcinomas of the forestomach of both sexes of F344 rats and B6C3F1 mice. The present studies provide a greater insight into the mechanism of the chronic toxicity of EtAc, valuable information about the structure toxicity relationships of all acrylic acid esters, and indicate a role of metabolic activation in the acute gastric toxicity caused by these important industrial chemicals.

PUBLICATIONS

Ghanayem, B. I., Maronpot, R. R. and Matthews, H. B. (1985) Ethyl acrylate-induced gastric toxicity I. Effect of single and repetitive dosing. Toxicol. Appl. Pharmacol. (In press).

Ghanayem, B. I., Maronpot, R. R. and Matthews, H. B. (1985) Ethyl acrylate-induced gastric toxicity II. Structure-toxicity relationships and mechanism. Toxicol. Appl. Pharmacol. (In press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21060-01 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Ethyl Acrylate Metabolism and the Metabolic Basis of Gastric Toxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Burhan I. Ghanayem
H. B. MatthewsStaff Fellow TRTP, NIEHS
Research Chemist TRTP, NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, N C 27709

TOTAL MAN-YEARS

0.3

PROFESSIONAL

0.3

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The absorption, distribution, covalent binding and excretion of 2,3-¹⁴C-ethyl acrylate (EtAc) was studied in F344 male rats. EtAc was readily absorbed, distributed to all tissues, metabolized and excreted mainly in the expired air as ¹⁴C-CO₂ (≈70% of the dose in 24 hours). 3-5% of the administered dose was excreted in the urine in 4 hours as mercapturic acids of EtAc and acrylic acid. Approximately 4% of the administered dose were excreted in the bile in 6 hours. The highest concentrations of radioactivity were found in the stomach, liver and kidneys respectively. Chemical fractionation of the forestomach and liver revealed that a major portion of the radioactivity in the stomach and liver was covalently bound to the protein fraction at 4 hours after treatment. Twenty-four hours after treatment, there was a significant decline in EtAc covalent protein binding in the liver, while there was no such decline in the stomach. No significant binding to nucleic acids was found in the stomach or the liver.

PROJECT DESCRIPTION

METHODS EMPLOYED: This work utilized ^{14}C -labeled EtAc in order to quantitate absorption, distribution, metabolism and clearance of EtAc in F344 male rats. Tissue distribution was quantitated using a tissue oxidizer and scintillation counting techniques. Covalent binding of EtAc to chemical fractions of tissues (lipids, proteins and nucleic acids) was performed by a series of extractions and centrifugations. Urine was analyzed by high performance liquid chromatography for EtAc metabolites.

MAJOR FINDINGS AND PROPOSED COURSE: EtAc was readily absorbed from the stomach ($\approx 90\%$ of the dose in 4 hours) of F344 male rats, distributed to all tissues examined, and rapidly excreted in the expired air and in the urine. EtAc did not bind to nucleic acids but did bind covalently to the protein of the forestomach and liver. The covalently bound radioactivity in the liver proteins declined significantly between 4 and 24 hours while no significant decline was seen in the covalent binding of EtAc derived radioactivity forestomach proteins. The major urinary metabolites of EtAc were the mercapturic acids of EtAc and acrylic acid.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Ethyl acrylate is a major industrial chemical (250 million pounds/yr) which has been identified as a potential carcinogen in a recent NTP bioassay. The present investigation is designated to determine the fate of EtAc in the intact animal. This study identifies possible mechanisms of EtAc toxicity under conditions of the NTP bioassay and provides data which will facilitate extrapolation of NTP chronic toxicity results to human exposure conditions. These results can be used to develop a risk/benefit ratio for EtAc and identify those exposure conditions which are most likely to threaten human health.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 21070-02 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

TCDD Teratogenicity: Modulation in Mixtures

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Linda S. Birnbaum	Research Microbiologist	TRTP	NIEHS
Others: James C. Lamb	Research Biologist	TRTP	NIEHS
James D. McKinney	Research Chemist	LMB	NIEHS
Martha Harris	Head Technician	TRTP	NIEHS
Robert M. Pratt	Research Biologist	LRDT	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 2709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

2.3

0.8

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

TCDD (dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin) is one of the most toxic chemicals known to man. Progressive weight loss and thymic atrophy are two of its most frequent toxic symptoms. The induction of cleft palate and hydro-nephrosis characterize the teratogenic response of mice to TCDD. Because of the sensitivity of this response, we decided to use teratogenicity to measure the interaction of TCDD and other compounds with which it occurs in the environment. Such chemicals include polychlorinated dibenzofurans, polychlorinated biophenyls, hormones such as thyroxins and hydrocortisone, and drugs. The effects on TCDD toxicity are dependent upon chemical structure and may support a mechanistic hypothesis of TCDD toxicity.

PROJECT DESCRIPTION

METHODS EMPLOYED: Female C57BL/6N (B6) mice are mated overnight with proven breeder male B6 mice. The presence of a vaginal plug defines gestation day 0. The pregnant mice are treated orally once a day on days 10-13 with the compounds of interest. On day 18, the dams are sacrificed, and the fetuses examined for cleft palate and kidney anomalies.

MAJOR FINDINGS AND PROPOSED COURSE: Hydronephrosis (HN) is the most sensitive teratogenic response to TCDD and related compounds in sensitive strains of mice. At doses where cleft palate incidence is 0-10%, all the fetuses have affected kidneys. A dose of 1 g/kg/day on gestation days 10-13 results in approximately 20% HN and no cleft palate. At 3 g/kg, the incidence of HN is essentially 100% while the cleft palate incidence is 5-8%. As the dose of TCDD is raised to 4, 5, or 6 g/kg/day, the incidence of cleft palate is approximately 40%, 60-70%, and 80-100%. This very steep dose response is also observed with TCDF and other related compounds.

The nature of the teratogenic interaction between TCDD and TCDF was shown to be additive. One g of TCDD is approximately equal to 30 g of TCDF in the cleft palate assay. The interaction between TCDD and 2,3,4,5,3',4'-hexachlorobiphenyl (HCB), a relatively non-toxic, mixed inducer, PCB, is also additive. Combination of 20 mg/kg HCB and 3 g/kg TCDD resulted in 40% cleft palate. While 20mg/kg HCB by itself failed to cause cleft palate, it did cause a low incidence of HN. Higher doses of HCB did produce cleft palate: 60 mg/kg caused about 3% cleft palate. Thus 1 g of TCDD is approximately equal to 20 mg of HCB.

Thyroid hormones seem to potentiate the teratogenicity of TCDD. Both T₃ and T₄ (thyroxine) enhance the induction of cleft palate. T₃ is five times more potent than T₄. Treatment of pregnant mice with 250 g T₃/kg and 3 g/kg TCDD increased the incidence of cleft palate five times over that seen with TCDD alone. Thyroid hormones themselves did not cause hydronephrosis or dioxin-like cleft palate.

Hydrocortisone (HC), an adrenal glucocorticoid, can also cause cleft palate. However, the mechanism of HC-induced cleft palate involves generalized growth retardation as opposed to the failure of programmed cell death involved in dioxin-induced cleft palate. When doses which are at the threshold for cleft palate induction, when given alone, i.e. 3 g/kg TCDD and 25 mg/kg HC, are given in combination, all the fetuses are affected. This suggests a potent synergism. However, morphometry of the palatal shelves late on gestation day 14 reveal that in the combination-treated fetuses, the shelves look HC-like, not dioxin-like. The mechanism of these interactions is under investigation.

The ability of chemicals to antagonize TCDD toxicity will also be studied. Such compounds include thyroid antagonists, rT₃, and certain anti-inflammatory drugs 2,4,6-triiodophenol, a potential thyroid antagonist, had no effect on the TCDD induction of cleft palate.

The nature of the interaction with other xenobiotics with which TCDD is found in the environment will also be examined.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: TCDD is an extremely toxic chemical of great environmental concern. It is found in combination with many other chemicals. The toxicity of such mixtures needs to be studied in an organized manner for predictive purposes and to help elucidate the mechanism of its toxicity.

PUBLICATIONS:

Birnbaum, L.S., Weber, H., Harris, M.W., Lamb, J.C., and McKinney, J.D.: Toxic interaction of specific polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin: Increased incidence of cleft palate in mice. Toxicol. Appl. Pharmacol. 77: 292-302, 1985.

Weber, H., Harris, M. W., Haseman, J.K., and Birnbaum, L. S.: Teratogenic potency of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), and TCDD-TCDF combinations in C57Bh/6N mice. Tox. Letts. (In press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 21075-02 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Xenobiotic Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Leo T. Burka	Research Chemist	TRTP	NIEHS
Others:	Burhan I. Ghanayem	Staff Fellow	TRTP	NIEHS
	C. P. Kool	Research Chemist	TRTP	NIEHS
	Richard Smith	Staff Fellow	TRTP	NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

1.7

PROFESSIONAL

0.9

OTHER

0.8

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Four compounds either being tested or being considered for testing by the NTP were investigated. Two major urinary metabolites of 1,2-dihydro-2,2,4-trimethylquinoline were identified as the O-sulfate conjugate of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline. The chemical disposition and to some extent the covalent binding of 1-chloro-2-methylpropene and 3-chloro-2-methylpropene were investigated. The most noteworthy observation in these studies is that about 33% of the dose of 1-chloro-2-methyl propene is exhaled, unmetabolized, in the first 6 hr. The major urinary metabolite of 3-chloro-2-methylpropene was found to be N-acetyl-S-(2-methylprop-2-enyl)-L-cysteine. It was demonstrated that the naturally-occurring flavone, kaempferol, was hydroxylated to give quercetin in vivo.

PROJECT DESCRIPTION

METHODS EMPLOYED: Metabolites were identified in biological samples using a high performance liquid chromatograph (HPLC) equipped with a variable wavelength UV detector and a radioactive flow detector. The chemical structures of the metabolites were determined either by isolation using a preparative HPLC column followed by nuclear magnetic resonance (NMR) and/or mass spectrometry (MS) or by coelution on HPLC with an authentic sample. In some cases the metabolite was chemically synthesized as further proof of structure. Chemical disposition studies were carried out in glass metabolism cages; tissue radioactivity was determined by oxidation to CO₂ and liquids instillation counting.

MAJOR FINDING AND PROPOSED COURSE:

- a) Major urinary metabolites of 1,2-dihydro-2,2,4-trimethylquinoline (TMQ) in the rat - Two TMQ metabolites comprising 50 and 30 percent of the total urinary radioactivity were isolated and purified using a reversed phase comparative HPLC column with ammonium acetate buffer (Ph5)-acetonitrile as the eluent. The NMR spectra of the metabolites were obtained at 300 MHz in deuterium oxide. Exact mass determinations were obtained by FAB MS/MS. The spectral data were most consistent with the major metabolite being either a 6-or 7-oxygenated derivative of TMQ. The metabolite was conclusively identified as the O-sulfate conjugate of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline by chemical synthesis. p-Anisidine was condensed with acetone using iodine as a catalyst to give 1,2-dihydro-6-methoxy-2,2,4 trimethylquinoline. The methoxy group was converted to the free phenol using boron tribromide and the phenol was subsequently converted to the O-sulfate by reaction with sulfur trioxide-pyridine complex. The HPLC retention time, UV spectrum and NMR spectrum of the synthetic product was identical to the isolated compound. The NMR and mass spectra of the second metabolite were consistent with a mono-O-sulfate conjugate of 1,2-dihydro-1,6-dihydroxy-2,2,4-trimethylquinoline. The sulfate group is probably on the phenol oxygen rather than the N-oxide, but this cannot be shown conclusively by the spectra alone. Attempted preparation of the 6-O-sulfate-N-hydroxy compound by chemical oxidation was unsuccessful.
- b) Chemical disposition studies on chloromethylpropenes - The tissue distribution and excretion profiles of [¹⁴C]-3-chloro-2 methyl propene (DMVC) and [¹⁴C]-1-chloro-2-methylpropene (MAC) were determined at a single time point (6 hr) and at one oral dose (150 mg/kg, corn oil) in male rats. In both cases the highest tissue concentration of radioactivity was found in the kidney forestomach and large intestine. A major part of the dose was excreted in 6 hr-- 35% of MAC, mostly in the urine (21%) and 57% of DMVC volatiles predominantly as expired volatiles (34%). The DMVC volatiles were trapped on activated carbon filters, analyzed by HPLC, and found to be 95% DMVC and 4% of a second, less polar compound that was not MAC.

There were four major urinary metabolites of MAC. The predominant one (70-80% of the total) was identified by its nmr spectrum as a mercapturic acid conjugate, N-acetyl-S-(2-methylprop-2-enyl)-L-cysteine. The identity was substantiated by chemical synthesis from N-acetyl-L-cysteine and MAC in aqueous sodium bicarbonate.

There were five major urinary metabolites of DMVC. The predominant one (35-40% of the total radioactivity) was investigated. It is a very polar compound, eluting from a reversed phase HPLC column in 99% H₂O-1% AcOH and has a strong UV absorption at 270nm. ¹H and ¹³C NMR spectra indicate the presence of a cysteine moiety. Signals at 7.5 ppm in the ¹H NMR and at 125 and 140ppm in the ¹³C NMR are probably due to an aromatic, possibly heterocyclic, moiety. Thus far we have been unable to obtain a mass spectrum using a number of techniques and the compound remains unidentified.

Covalent binding of DMVC and MAC to calf thymus DNA after activation by rat liver microsomes was investigated. No appreciable binding to DNA was observed with DMVC or MAC. The microsomes were producing an active species, however, since there was a 7-fold increase in covalent binding to microsomal protein in the presence of NADPH (18 vs 2.5 pmol eq./ g protein) with DMVC and a two fold increase (96 vs 48 pmol eq./ g protein) with MAC.

The distribution of radioactivity in liver and forestomach tissue from animals treated with [¹⁴C] DMVC and [¹⁴C] MAC was investigated. The greatest concentration of radioactivity in the nucleic acid fraction, 400 pmol eq./g of tissue was found in the forestomachs of rats treated with MAC. All tissues showed appreciable protein binding ranging from 13% (DMVC, liver) to 80% (DMVC, forestomach) of the total radioactivity in the tissue.

- c) The *in vivo* metabolism of kaempferol to quercetin - The *in vivo* conversion of kaempferol, a compound selected for possible study by the NTP to quercetin, a known mutagen already under study by the NTP, was carried out. Kaempferol was administered by i.v. injection to F-344 rats anesthetized with pentobarbital and with the common bile duct cannulated.

The bile was collected over a 6 hr period treated with HCl (2N) and analyzed by HPLC using a reversed phase column with water-acetonitrile-tetrahydrofuran (3:1:1) as the eluent. Quercetin was identified by coelution with an authentic sample and by identity of the UV spectrum (max 370nm) with an authentic sample.

The study of metabolism of xenobiotics on test or being considered for testing in the NTP will continue. Specific compounds to be investigated in the coming year will be determined by the needs and interests of the program.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Toxic xenobiotics require metabolism by the organism before the toxicity or carcinogenicity often xenobiotic is elicited. It is therefore important to know what metabolites are formed from a particular xenobiotic, not only to determine if the metabolite itself is the toxic agent, but also to evaluate the possibility that an intermediate in the formation of the metabolite may be responsible for all or part of the toxic effect. Identified metabolites form a useful data base from which predictions can be made as to site and extent of metabolism in other compounds and also to indicate which metabolizing enzymes may need further study. Identification of metabolites from different species or sexes frequently

provides an explanation of specific toxicity observed in the respective species or sex. And finally, elucidation of mechanisms of toxicity observed in laboratory animals facilitates extrapolation of laboratory data to man and the assessment of risk/benefit ratios involving human exposure.

PUBLICATIONS

Ravindranath, V., Burka, L. T., and Boyd, M. R.: Syntheses of 2-([¹⁴C]methyl)furan and 4-OXO[5-¹⁴C]-2-pentenal. J. Labelled Compds. and Radiopharmaceuticals, 11: 713-718, 1984

Ganguli, M., Burka, L. T., and Harris, T. M.: Structural studies of the mycotoxin verrucosidin. J. Org. Chem. 49: 3762-3766, 1984.

Boyd, M. R., Ravindranath, V., Burka, L. T., Dutcher, J. S., Franklin, R. B., Strathan, C. N., Haschek, W. M., Hakkinen, P. J., Morse, C. C., and Witschi, H. P.: Drug Metabolizing enzyme systems and their relationship to toxic mechanisms. In Li, A. P. (ed.): Toxicity Testing New Approaches in Human Risk Assessment. New York, Raven Press, 1985, pp. 119-127.

Burka, L. T., Guengerich, F. P., Willard, R. J., Macdonald, T. L.: Mechanism of cytochrome P-450 catalysis. Mechanism of N-dealkylation and amine oxide deoxygenation. J. Am. Chem. Soc. 107: 7549-2551, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 30044-09 STB
PERIOD COVERED October 1, 1984 through September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Toxicology of Environmental Chemicals		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> P.I. E.W. Van Stee OTHERS: Michael P. Moorman Richard A. Sloane </div> <div style="width: 50%;"> Veterinary Officer TRTP (NTP) NIEHS Engineering Officer TRTP (NTP) NIEHS Biologist TRTP (NTP) NIEHS </div> </div>		
COOPERATING UNITS (if any) Northrop Services, Incorporated		
LAB/BRANCH Systemic Toxicology Branch		
SECTION Inhalation Toxicology		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS .5	PROFESSIONAL .1	OTHER 0.4
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div style="width: 30%;"> <input type="checkbox"/> (b) Human tissues </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) Exposure of strain A mice for 6 months to vinyl chloride, ethylene dibromide or ethylene oxide, cause concentration-related increases in numbers of pulmonary adenomas that were formed. This inexpensive model may be useful in helping to identify inhalant carcinogens. Mice were exposed to nitrogen dioxide following profiles with equal integrals of concentration with time but different maximum concentrations. The wet/dry lung weights were taken as a measure of pulmonary edema. The same study has been conducted with Acrolein as the exposure compound.		

PROJECT DESCRIPTION

OBJECTIVE: The objective of this project is to study the effects of toxicologically significant compounds administered by inhalation.

METHODS EMPLOYED: 1. Female Strain A mice were exposed to inhalation 6 hr/da, 6 days/week for six months to different concentrations of carbon disulfide, naphthalene, ethylene dibromide, vinyl chloride and ethylene oxide. 2. Three groups of female CD-1 mice were exposed by inhalation to 10, 20, and 40 ppm of nitrogen dioxide, 6, 3, and 1.5 hours respectively, 5 days/week for six weeks. Mice from each exposure group and a control group were sacrificed on Friday of each week and the lung/trachea tissue excised. The ratio of dry to wet weights was used as a measure of pulmonary edema. 3. Three groups of female CD-1 mice were exposed by inhalation to 2, 4, and 8 ppm of acrolein, 6, 3, and 1.5 hours respectively, 5 days/week for six weeks. Mice from each exposure group and a control group were sacrificed on Friday of each week and the lung/trachea tissue excised. The ratio of dry to wet weights was used as a measure of pulmonary edema.

MAJOR FINDINGS AND PROPOSED COURSE: 1. The exposure of female, Strain A/J mice to vinyl chloride, ethylene dibromide, or ethylene oxide was accompanied by the formation of increased numbers of pulmonary adenomas in proportion to the exposure concentration. Exposure to carbon disulfide or naphthalene did not cause excess tumor formation. Papers on these studies are being written by the contractor's project director with input from the project officer. 2. The results of this study are not yet available. 3. The results of this study are not yet available.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Chemicals like vinyl chloride, ethylene dibromide, and ethylene oxide that are known to be animal carcinogens and/or mutagens. A test model using Strain A/J mice is potentially useful as a "screen" for predicting carcinogenesis. These chemicals were tested to evaluate the ability of the test model to accurately detect known animal carcinogens.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 30106-11 STB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders) The Effects of Environmental Pollutants on the Immune System		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI: Michael I. Luster	Research Microbiologist	STB NIEHS
Others: G. Boorman A. Tucker K. Korach T. Eling	Veterinary Medical Officer IPA, Medical College of Virginia Research Chemist Research Chemist	CPB NIEHS STB NIEHS LRDT NIEHS LMB NIEHS
COOPERATING UNITS (if any) Laboratory of Reproductive & Developmental Toxicology, IRP Laboratory of Molecular Biophysics, IRP		
LAB/BRANCH Systemic Toxicology Branch, TRTP		
SECTION Immunotoxicology		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS 4.25	PROFESSIONAL 1.5	OTHER 2.75
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.) The ongoing objectives of the immunological-toxicology group include the following interrelated efforts: (1) to <u>evaluate and examine the influence of selected environmental chemicals on the immune response</u> including cellular changes associated with chemical interactions in lymphoreticular cells; (2) to <u>relate alterations in immunological functions with both general toxicity as well as specific organ toxicity</u> ; (3) to relate changes in immunological functions with <u>altered host resistance</u> following challenge with either syngeneic tumor cells or infectious agents employing a defined panel of <u>infectivity models</u> ; and (4) to <u>refine and validate a panel of immune and host resistance procedures in order to better define immunotoxicity</u> and correlate changes in immune function with <u>altered host resistance</u> . This approach should potentially allow for more accurate assessment of <u>human health risk</u> as well as determine <u>no-effect levels for immunotoxic chemicals</u> .		

PROJECT DESCRIPTION

METHODS EMPLOYED: The assays employed to assess immunological dysfunction or altered host resistance following chemical exposure are listed in Table 1.

Table 1
Comprehensive Panel for Defining Immune
Alterations Currently Being Employed at NIEHS

Parameter	Procedure Performed
Pathotoxicology	Hematology Profile - hemoglobin, red blood cell count, white blood cell count, differential Weights - body, spleen, thymus, liver, kidney Histology - liver, thymus, adrenal, lung, kidney, heart, spleen
Host Resistance	Tumor Assays - tumor cell challenge TD ₁₀₋₂₀ and radiometric tumor mass <u>Listeria monocytogenes</u> LD ₁₀₋₂₀ challenge Streptococcus challenge LD ₁₀₋₂₀ challenge Plasmodium induced parasitemia
Marker Enumeration	Splenic B-cell, T-cell and Lyt enumeration
Delayed Hypersensitivity	Radiometric assay with T-cell dependent antigen
Lymphocyte Proliferation	Mixed leukocyte culture Mitogens - PHA, Con A, LPS
Humoral Immunity	<u>In vivo</u> and <u>in vitro</u> antibody response to T-dependent (SRBC), T-independent (LPS), B1 (TNP-LPS) and B2 (TNP-Ficoll) antigens
Macrophage Function ¹	Resident peritoneal cell numbers and differential Phagocytosis Ectoenzymes - 5'-nucleotidase, leucine amino peptidase and alkaline phosphatase
Natural Killer Cell Activity	<u>In vitro</u> cytotoxicity using YAC-1 tumor cells

Bone Marrow Colony
Forming Units

CFU-S - (hematopoietic stem cell proliferation)

CFU-GM - (granulocyte/macrophage progenitor proliferation)

⁵⁹Fe - Incorporation into the bone marrow

Cellularity

¹Employs both resident peritoneal cells and MAF activated macrophages.

MAJOR FINDINGS AND PROPOSED COURSE: The mechanisms of estrogen immunoregulation have been a major area of investigation by our group in recent years. The biological relevance of these observations was examined in a series of host infectivity models. Several compounds with estrogenic activity and defined immunological effects caused an increased susceptibility of mice to the parasite, Toxoplasma gondii, as measured by brain cyst formation. Host resistance to this parasite was altered by pharmacological, but not physiological, levels of estrogens. The mechanism of increased susceptibility to infection with Listeria monocytogenes was also examined. Interleukin 2 production by splenic lymphocytes from estrogen-treated mice was decreased, which resulted in impaired proliferation of antigen-sensitized T lymphocytes which are required for recovery. These studies are now complete.

The mechanism of immunosuppression by benzidine remains an area of intense investigation. Lymphocyte responses appear to be suppressed as a result of alterations in arachidonic acid metabolism. Present studies are focused on quantifying these alterations, as several metabolites of arachidonic acid are potent inhibitors of lymphocyte activation, whereas others are required for stimulating guanyl cyclase, which is essential for lymphocyte activation. Bone marrow stem cells are also a sensitive target for benzidine immunosuppression, and the pre-B cell (CFU-BL) is also being studied with respect to the role of arachidonic acid oxidation in cellular proliferation. Preliminary data indicates that the lipoxygenase pathway is essential for cellular responses and is selectively affected by benzidine.

A major area of investigation in this laboratory centers on the alteration of stem cell and B cell differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). We have several in vitro models of differentiation (CFU-GM response to colony stimulating factor and B cell maturation to antibody-producing cells) which are suppressed by TCDD. The suppression is mediated by the interaction of TCDD with the Ah receptor, as evidenced by genetic and structure-activity studies. Using the CFU-GM model, an antagonist, 1-amino-trichlorodibenzo-p-dioxin, has been identified and appears to block TCDD toxicity at the receptor level. The relationship between thyroid hormones and TCDD toxicity is also being examined in this model. Combinations of T3 and T4 reduce CFU-GM cells, and this can be blocked by the 1-amino antagonist. The role of the Ah receptor in B cell differentiation is being examined by studying events associated with B cell activation, such as membrane depolarization, increased Ia expression, protein kinase C activation, phospholipid metabolism, and expression of growth receptors. Receptor expression is cell cycle dependent, thus cell cycle studies are an integral part of this work.

Studies are now underway involving perinatal exposure of mice to ethylene glycol monomethyl ether. Thymic atrophy has previously been observed in animals exposed to this chemical, and the present studies are designed to examine the consequences of exposure during fetal and postnatal development of immune competence.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A correlation has been clearly established between the administration of chemical immunosuppressants and an increased incidence of infectious diseases and neoplasia. The evidence for increased bacterial, viral, fungal and parasitic diseases in patients on chronic immunosuppressive chemicals has been well documented by Allen (Infection complicating neoplastic disease and cytotoxic therapy. In Infection and the Compromised Host, 1976). Likewise, McKhann (Transplantation 8:209, 1971) and more recently Penn (In Current Problems in Cancer, Vol. 6, 1982) observed that the incidence of cancer in renal transplant recipients on prolonged immunosuppressive chemotherapy was higher than in the general population. In another light epidemiological evidence has indicated that immunotoxic chemicals may act as predisposing agents in patients who develop Acquired Immunodeficiency Syndrome (e.g. Goedert et al., Lancet, 1982).

Studies in laboratory animals also have supported these clinical observations and demonstrated an enhanced incidence of UV-induced or benzopyrene-induced cancer in mice treated with immunosuppressive agents. The mechanisms and relationship between altered host resistance and immune dysfunction is complex, poorly defined and of extreme importance. Chemicals of environmental concern have been recently shown to induce immunosuppression as evidenced by depressed antibody mediated immunity, cell-mediated immunity or M ϕ dysfunction in rodents following sublethal exposure. Some of the chemicals which induce immunologic effects in rodents include 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated biphenyls, polybrominated biphenyls, gallic acid, DES, BP, hexachlorobenzene, pentachlorophenol, certain organo and heavy metals. Some studies have indicated that exposure to certain chemicals can alter resistance to bacteria, viruses, parasites and transplantable tumor cells. Of major concern is the correlation of these immunologic findings with altered host susceptibility and the extrapolation of these chemically-induced immunobiologic effects to humans. Furthermore, in order to accurately predict human health risk, no effect levels and structure-activity relationships, the mechanisms of immunotoxicity need to be more clearly defined.

PUBLICATIONS

Luster, M.I., Boorman, G.A., Korach, K.S., Dieter, M.P., and Hong, L.: Mechanisms of estrogen-induced myelotoxicity: Evidence of thymic regulation. Int. J. Immunopharmacol. 6: 287-297, 1984.

Luster, M.I., Hayes, H.T., Korach, K., Tucker, A.N., Dean, J.H., Greenlee, W., and Boorman, G.A.: Estrogen immunosuppression is regulated through estrogenic responses in the thymus. J. Immunol. 133: 110-116, 1984.

Luebke, R.W., Luster, M.I., Dean, J.H., and Hayes, H.T.: Altered host resistance to Trichinella spiralis infection following subchronic exposure to diethylstilbestrol. Int. J. Immunopharmacol. 6: 609-617, 1984.

Luster, M.I., Tucker, A.N., Hong, L., Boorman, G.A., and Patterson, R.: In vivo and in vitro effects of TCDD on stem cell and B cell differentiation. In Poland, A. and Kimbrough, R. (Eds.): Biological Effect of Dioxin. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p. 411-420, 1984.

Pung, O.J., Luster, M.I., Hayes, H.T. and Rader, J.: Influence of steroidal and nonsteroidal sex hormones on host resistance in the mouse: Increased susceptibility to Listeria monocytogenes following exposure to estrogenic hormones. Infect. and Immunity 46: 301-307, 1984.

Pung, O.J., and Luster, M.I.: Toxoplasma gondii: Estrogens alter resistance to infection in mice. Exp. Parasitol., in press.

Tucker, A.N., Hong, L., Boorman, G.A., Pung, O.J., and Luster, M.I.: Alteration of bone marrow cell cycle kinetics by diphenylhydantoin: Relationship to folate utilization and immune function. J. Pharmacol. Exp. Ther., in press.

Luster, M.I., Tucker, A.N., Hayes, H.T., Pung, O.J., Burka, T., McMillan, R., and Eling, T.: Immunosuppressive effects of benzidine in mice: evidence of alterations in arachidonic acid metabolism. J. Immunol., in press.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 70200-11 STB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Mechanisms for Regulating for Intracellular Bioavailability of Metals

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	B. A. Fowler	Research Biologist	STB	NIEHS
Others:	P. Mistry	Visiting Fellow	STB	NIEHS
	P. Goering	NRSA Postdoctoral Fellow	STB	NIEHS
	G. DuVal	NRSA Postdoctoral Fellow	STB	NIEHS

COOPERATING UNITS (if any)

C. F. Chignell, Laboratory of Molecular Biophysics

LAB/BRANCH

Systemic Toxicology Branch

SECTION

Chemical Disposition

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

4.5

2.5

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Molecular mechanisms which regulate the intracellular bioavailability of metals such as lead and cadmium have been studied. High affinity cytosolic lead-binding proteins (PbBP) of 63,000 (63K) and 11,500 (11.5K) daltons from kidneys of rats have been partially purified by gel and anion exchange chromatography, electrophoresis, and sucrose density gradient analysis. These molecules were found to exhibit dissociation constants (K_d) for lead of $10^{-8}M$. Competitive binding studies on sucrose density gradients with cytosol showed displacement of ^{203}Pb by Pb^{11} , Cd^{11} , and Zn^{11} ions but not Ca^{11} ions. Cell-free nuclear translocation studies showed both time- and temperature-dependent uptake. Cd^{11} and Zn^{11} ions effectively blocked the nuclear uptake of ^{203}Pb . In vivo Pb-injection studies showed a close temporal relationship between formation and loss of Pb intranuclear inclusions in renal proximal tubule cell nuclei and marked changes in renal 2-D gel ^{35}S -labelled protein synthesis patterns. The data indicate that these high affinity PbBP, which act as the initial cytosolic ligands for Pb in the kidney, are capable of mediating the intranuclear translocation of Pb and that the presence of Pb within renal nuclei is temporally associated with marked changes in renal gene expression. The 11.5K dalton protein, but not the 63K protein, was also found to regulate the inhibitory effects of Pb on the heme biosynthetic pathway enzyme -aminolevelinic acid dehydratase (ALAD). Reversal of Pb-induced inhibition of hepatic ALAD activity was dependent on the concentration of 11.5K dalton PbBP added to the reaction mixture. The data indicate that the 11.5K dalton protein confers partial resistance to Pb inhibition of liver ALAD in vitro and suggests a similar role for this protein in kidney with respect to the resistance of renal ALAD to Pb inhibition. PbBP chelation of Pb and donation of Zn to the ALAD were found to be the mechanisms of this effect.

PROJECT DESCRIPTION

METHODS EMPLOYED : Methods and techniques employed for these studies include conventional gel permeation and ion exchange chromatography as well as high performance liquid chromatography for isolation and purification of the PbBP's. Saturation and Scatchard analysis have been used to assess ^{203}Pb binding affinities for these molecules. Sucrose density gradient analysis has been used for metal competition and cell-free nuclear translocation studies. Measurement of δ -aminolevulinic acid dehydratase is used as a sensitive and specific biological indicator of Pb bioactivity and as an endpoint for assessing PbBP regulation of this metal.

MAJOR FINDINGS:

- 1) The Pb^{11} binding characteristics of the previously reported Pb^{11} binding proteins of rat kidney cytosol were investigated further. Saturation and Scatchard analysis of ^{203}Pb binding in whole cytosol and in 40% saturated ammonium sulfate precipitated fractions disclosed a class of relatively high-affinity sites with an apparent K_d of approximately 10^{-8} M. Two ^{203}Pb binding proteins with approximate molecular masses of 63K and 11.5K daltons and a high molecular weight component ($>200\text{K}$) were isolated by Sepharose-6B column chromatography. The time course of association of ^{203}Pb with cytosol and the 63K protein showed maximum binding at 18 hr which was stable up to 25 hr at 40°C . The approximate half-time dissociation rate ($T_{1/2}$) of specifically bound ^{203}Pb to the 63K protein was 100 min at 40°C whereas the 11.5K protein showed little dissociation of specifically bound ligand at this temperature. Saturation analysis of the three isolated proteins disclosed low capacity, high-affinity sites with similar apparent K_d values to the cytosol assay. Sucrose density gradient analysis of kidney cytosol showed approximate sedimentation coefficients of 2S, 4.6S and 7S for the 11.5K, 63K and the high molecular weight proteins, respectively. Competitive binding studies with cytosol demonstrated displacement of ^{203}Pb by Pb^{11} , Cd^{11} and Zn^{11} ions but not Ca^{11} ions. Nuclear uptake studies showed both time- and temperature-dependent uptake of ^{203}Pb from kidney cytosol and KCl extraction data indicated that these high-affinity lead binding proteins which act as the initial cytosolic ligands for Pb^{11} in the kidney are capable of mediating the nuclear uptake of Pb^{11} .
- 2) The bioavailability of Pb in kidney is mediated in part by binding to high affinity cytosolic Pb-binding proteins (PbBP) of 11,500 (11.5K) and 63,000 (63K) daltons, which are not found in liver. Renal δ -aminolevulinic acid dehydratase (ALAD) is also markedly more resistant to Pb inhibition than hepatic ALAD in vivo. The possible roles of PbBP in mediating this resistance were investigated. Rat liver or kidney cytosol was incubated with Pb over a concentration range of 0.1 to $10\mu\text{M}$. Renal ALAD was 7.5 times more resistant to Pb inhibition than that in liver. Kidney cytosol and ^{203}Pb were incubated before Sephadex G-75 or G-150 column chromatography to isolate the 11.5K and 63K PbBP, respectively. Inhibition of hepatic ALAD activity by Pb was partially reversed by a single addition of semipurified 11.5K PbBP in the presence of 0.1 to $0.4\mu\text{M}$ Pb, but no protective effect was observed at higher concentrations of Pb. This effect was not observed with the 63K PbBP added at an equivalent high affinity binding capacity or bovine serum albumin

added at an 3-fold higher total protein concentration. Reversal of Pb-induced inhibition of hepatic ALAD activity was dependent on the concentration of 11.5K PbBP in the reaction mixture. Kinetic analysis of either hepatic or renal ALAD activity at an IC_{50} concentration of Pb indicated a noncompetitive inhibition pattern. Addition of the semipurified 11.5K PbBP to the assay mixture markedly reduced the inhibitory effects of Pb on the V_{max} of the enzyme from either tissue. The data indicate that the 11.5K PbBP confers partial resistance to Pb inhibition of liver ALAD in vitro and suggests a similar role for this protein in kidney with respect to the insensitivity of renal ALAD to Pb inhibition. The molecular mechanisms of this protection were found to involve two phenomena involving both PbBP chelation of Pb and donation of Zn from PbBP to ALAD with subsequent activation of the enzyme. Similar results were obtained using PbBP isolated from brain and purified zinc metallothionein.

PROPOSED COURSE: The research efforts of this laboratory for the next several years are focused on purifying and characterizing PbBP in comparison with other known high affinity metal-binding proteins such as MT or scallop and oyster CdBP's, such that the relationships between protein amino acid sequence, secondary structure, and formation of the metal-binding sites may be understood. A second area of intense interest concerns further elucidation of the various functions played by these molecules in mediating the intracellular bioavailability/activity of metals such as Ga, As, Cd and Pb with respect to ultrastructural/biochemical manifestations of cell injury.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The significance of the above findings with respect to PbBP is that these data demonstrate a central role for these target tissue specific ligands in mediating the biological activity of this ubiquitous element, particularly at low dose levels. These molecules hence provide a molecular mechanism for explaining the nuclear translocation of Pb, interaction of Pb with DNA and subsequent genetic and possible carcinogenic responses associated with this element in the kidney. In addition, these proteins also clearly regulate the availability of Pb to sensitive enzymes (ALAD) in a highly dose-related manner indicating that previously reported tissue differences in ALAD susceptibility to Pb may now be explained on a molecular basis. The importance of this knowledge rests with providing a molecular basis for predicting tissue, age, and sex-related differences to Pb inhibition of sensitive biological indicators such as ALAD. The approach taken has broad applicability to understanding organ and cell-specific molecular factors which regulate metal-induced cell injury.

PUBLICATION

Goering, P. S., and Fowler, B. A.: Regulation of lead inhibition of δ -aminolevulinic dehydratase by a high affinity renal lead-binding protein. J. Pharmacol. Exp. Therap. 231: 66-71, 1984.

Victory, W. W., Miller, C. R., and Fowler, B. A.: Lead accumulation by rat renal brush border membrane vesicles. J. Pharmacol. Exp. Therap. 231: 589-596, 1984.

Mistry, P., Lucier, G. W., and Fowler, B. A.: High affinity lead-binding proteins from rat kidney cytosol: Mediate cell-free nuclear translocation of lead. J. Pharmacol. Exp. Therap. 232: 462-469, 1985.

Goering, P. L., and Fowler, B. A.: Mechanisms of renal lead-binding protein protection against lead-inhibition of delta-aminolevulinic acid dehydratase. J. Pharmacol. Exp. Therap. (In press).

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Fowler, B. A.: Indium. In Friberg, L. and Nordberg, G. F. (eds.): Handbook on the Toxicology of Metals. Elsevier/North Holland Publ. Co., Second Edition (In press).

Fowler, B. A., and Vouk, V. B.: Bismuth. In Friberg, L., and Nordberg, G. F. (eds): Handbook on the Toxicology of Metals. Elsevier/North Holland Publ. Co., Second Edition (In press).

ILLINOIS INSTITUTE OF TECHNOLOGY RESEARCH INSTITUTE
Chicago, Illinois 60616
(NIH-N01-ES-1-5000)

TITLE: Chemical-Induced Immunotoxicity

CONTRACTOR'S PROJECT DIRECTOR: Peter Thomas, Ph.D.

PROJECT OFFICER (NIEHS): M.I. Luster, Ph.D.
Immunotoxicology Group Leader, STB, TRTP

DATE CONTRACT INITIATED: February 1, 1981

CURRENT ANNUAL LEVEL: \$300,000

PROJECT DESCRIPTION

OBJECTIVES: The objective of this contract encompasses efforts to develop improved assay methodology for measuring altered host resistance and immunological impairment in rodents exposed to chemicals of environmental concern, interlaboratory assay validation, and evaluation of selected chemicals with respect to their ability to alter immune functions and host resistance to challenge with infectious agents or tumor cells. There are three major tasks involved in this project. They include: (i) evaluation of methods for evaluating host resistance to bacteria, viruses, animal parasites and transplantable tumors; (ii) establishment and proficient demonstration of a standardized set of immunologic tests; and (iii) integration and validation of the test systems for altered host resistance and immunological function using at least five chemicals selected by NIEHS.

METHODS EMPLOYED:

I. Host Resistance Assays

Altered susceptibility to challenge with various infectious agents are being examined in mice following exposure to immunotoxic chemicals of environmental concern including diethylstilbestrol, cadmium chloride and dimethylnitrosamine. A wide range of infectious agents are being employed for study and development as models, for which considerable information is available concerning the operative host resistance mechanisms. The original selected group of organisms include Listeria, Streptococcus, and Klebsiella as the three bacteria, influenza and Herpes simplex I as the two viruses, Trichinella spiralis as the parasite and the B16F10 as the transplantable tumor and more recently PYB6 tumor, Herpes simplex II and ³⁵S-Klebsiella pneumoniae, resistance models.

II. Immune Function Tests

The following immune function assays are evaluated: (1) lymphocyte proliferation to mitogens and allogenic leukocytes; (2) antibody plaque forming cell response to a T-cell-dependent antigen (both direct and indirect); (3) quantitation of serum immunoglobulin levels; (4) delayed hypersensitivity responses using radioisotopic assays; (5) assays for macrophage function including RES clearance, tumor cell cytostasis, enzyme activity and phagocytosis; (6) antibody response to a T cell-independent antigen; (7) natural killer cell activity.

III. Standard Toxicology

Evaluation of body weight, lymphoid organ weight, selected histopathology, hematology profile and activities of selected liver enzymes are included to relate the toxic effects of chemical exposure on immune dysfunction.

MAJOR FINDINGS AND PROPOSED COURSE: The contractors have made substantial progress in the development, validation and refinement of the immunologic and host resistance assays as originally outlined in the goals and milestones of the contract. A core panel for screening potentially immunotoxic chemicals has been developed (Table 1).

Table 1
Core Panel for Detecting Chemical-Induced Immunotoxicity

Parameter	Procedure Performed
Infectivity Models	<ul style="list-style-type: none">• Influenza challenge: LD₂₀ + LD₈₀ challenge• <i>Listeria monocytogenes</i>: LD₂₀ + LD₈₀ challenge• Herpes simplex virus II: LD₂₀ + LD₈₀ challenge• PYB6 tumor cell challenge: TD₂₀ + TD₈₀ challenge• <i>Trichinella spiralis</i> infection: percent expulsion
Immunological Assays	<ul style="list-style-type: none">• Natural killer cell activity• Delayed hypersensitivity response• Antibody response to T-dE9 (SRBC)• Mixed leukocyte cultures• Lymphocyte blastogenesis to mitogens
Toxicology Evaluation	<ul style="list-style-type: none">• Body weight and selected organ weights• Hematology• Selected serum chemistries
Macrophage Functions	<ul style="list-style-type: none">• Peritoneal cell numbers and differential• Phagocytosis• Ectoenzymes

The test assays have completed an interlaboratory validation phase and appear to be both sensitive and reproducible with regards to detecting chemical-induced immunotoxicity. Several compounds of specific interest to the National Toxicology Program have been examined in this period. In addition to developing a strong data base, these studies have been used to integrate and validate the test systems.

Since the last reporting period, studies were conducted to examine the immunomodulatory effects of vinylcyclohexene diepoxide (VCH) and of ethylene dibromide (EDB) as part of Phase 3 of this contract. In addition, studies of the effects of DMBA on host resistance to influenza and final experiments evaluating DMN and macrophage function were performed. Additional studies also were performed to optimize conditions for macrophage activation by lymphokine preparations. EDB was administered to B6C3F1 mice by gavage at dose levels of 0, 1, 2 or 3 mg/kg/day for 14 consecutive days. There were no statistically significant immunological effects as a result of chemical exposure. Female B6C3F1 mice were

administered VCH by dermal exposure at dose levels of 0, 1.25, 2.5 or 5.0 mg/kg/mouse/day for 14 days. There were no significant toxicological effects except a slight increase in kidney weights nor were there any hematological changes. VCH caused a marked depression of the humoral antibody response and a trend for increased cell mediated immunity. Consistent with this observation, exposed mice tended to resist herpes virus infection to a greater degree than control mice. These results indicated that VCH has a selective effect on immune functions.

This contract expired on February 15, 1985.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A correlation has been clearly established between the administration of chemical immunosuppressants and an increased incidence of infectious diseases and neoplasia. The evidence for increased bacterial, viral, fungal and parasitic diseases in patients on chronic immunosuppressive chemicals has been well documented by Allen (Infection complicating neoplastic disease and cytotoxic therapy. In Infection and the Compromised Host, 1976). Likewise, McKhann (Transplantation 8:209, 1971) and more recently Penn (In Current Problems in Cancer, Vol. 6, 1982) observed that the incidence of cancer in renal transplant recipients on prolonged immunosuppressive chemotherapy was 4.6-6.1 times higher than in the general population. In another light, evidence has suggested that immunotoxic chemicals may act as predisposing agents in patients who develop Acquired Immunodeficiency Syndrome (AIDS) (e.g. Goedert et al., Lancet, 1982).

Studies in laboratory animals also have supported these clinical observations and demonstrated an enhanced incidence of UV-induced or benzopyrene-induced cancer in mice treated with immunosuppressive agents. The mechanisms and relationship between altered host resistance and immune dysfunction is complex, poorly defined and of extreme importance. Chemicals of environmental concern have been recently shown to induce immunosuppression as evidenced by depressed antibody mediated immunity, cell-mediated immunity or M ϕ dysfunction in rodents following sublethal exposure. Some of the chemicals which induce immunologic effects in rodents include 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated biphenyls, polybrominated biphenyls, gallic acid, DES, BP, hexachlorobenzene, pentachlorophenol, certain organo and heavy metals. Some studies have indicated that exposure to certain chemicals can alter resistance to bacteria, viruses, parasites and transplantable tumor cells. Of major concern is the correlation of these immunologic findings with altered host susceptibility and the extrapolation of these chemically-induced immunobiologic effects to humans.

MEDICAL COLLEGE OF VIRGINIA/VIRGINIA COMMONWEALTH UNIVERSITY
Richmond, Virginia 23298
(NIH-N01-ES-1-5001)

TITLE: Chemical Induced Immunotoxicity

CONTRACTOR'S PROJECT DIRECTOR: Albert E. Munson, Ph.D.

PROJECT OFFICER (NIEHS): M.I. Luster, Ph.D.
Immunotoxicology Group Leader, STB, TRTP

DATE CONTRACT INITIATED: February 1, 1981

CURRENT ANNUAL LEVEL: \$262,000

PROJECT DESCRIPTION

OBJECTIVE: The objective of this contract encompasses efforts to develop improved assay methodology for measuring altered host resistance and immunological impairment in rodents exposed to chemicals of environmental concern, inter-laboratory assay validation and evaluation of selected chemicals to alter immune functions and host resistance to challenge with infectious agents or tumor cells. There are 3 major tasks involved in this project. They include: (i) evaluation of methods for evaluating host resistance to bacteria, viruses, animal parasites and transplantable tumors; (ii) establishment and proficient demonstration of a standardized set of immunologic tests; and (iii) integration and validation of the test systems for altered host resistance and immunological function using at least 5 chemicals selected by NIEHS.

METHODS EMPLOYED:

I. Host Resistance Assays

Altered susceptibility to challenge with various infectious agents is being examined in mice following exposure to chemicals of environmental concern. A wide range of infectious agents is being employed for study and development as models, for which considerable information is available concerning the operative host resistance mechanisms. The original selected group of organisms were EMC and herpes simplex type 2 viruses; S. pneumoniae, E. coli, and L. monocytogenes as the bacteria; P. berghei as a parasite and the B16 melanoma as the transplantable tumor.

II. Immune Function Tests

Evaluation of the following immune function assays are underway: (1) lymphocyte proliferation to mitogens and allogenic leukocytes; (2) antibody plaque forming cell response to a T cell-dependent antigen (both direct and indirect); (3) quantitation of serum immunoglobulin levels; (4) delayed hypersensitivity responses using radioisotopic assays; (5) assays for macrophage function including RES clearance, tumor cell cytostasis, enzyme activity and phagocytosis.

III. Standard Toxicology

Evaluation of body weight, lymphoid organ weight, selected histopathology, hematology profile and activities of selected liver enzymes are included to relate the toxic effects of chemical exposure on immune dysfunction.

MAJOR FINDINGS AND PROPOSED COURSE: The contractors have made substantial progress in the development, validation and refinement of the immunologic and host resistance assays as originally outlined in the goals and milestones of the contract. The test assays have completed an interlaboratory validation phase and appear to be both sensitive and reproducible with regards to detecting chemical-induced immunotoxicity. Several compounds of specific interest to the National Toxicology Program have been examined in this period. In addition to developing a strong data base, these studies have been used to integrate and validate the test systems.

1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin (HCDD) was selected to be investigated for effects on the immune system and host resistance because it was a major contaminant of pentachlorophenol which was shown to have immunosuppressive effects. The doses of HCDD were 0.056, 0.56, and 5.6 $\mu\text{g/kg}$ administered daily for 14 days by gavage. 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) was used as a comparative control. B6C3F1 female mice exposed to HCDD or TCDD showed no effects on body weight or body weight gain and the animals appeared normal throughout the experimental period. Selected immunopathological studies showed that the major target organ for HCDD was the liver. The microsomal parameters associated with cytochrome P450 were increased as expected. Spleen and thymus weights were slightly decreased. Hematologic parameters were not changed from the control group. HCDD produced several perturbations on the immune system of mice. The number of splenic T lymphocytes was increased in HCDD exposed mice. The spleen IgM and IgG response was reduced to 16% of control, the DHR to KLH was reduced to 53% of control, and the MLR was reduced to 71% of control. There was no effect on NK cell activity, but there was a decrease in serum complement. The only effect seen on macrophage function was a decrease in hepatic phagocytosis. The lowest effect level for the immunological studies was 0.56 $\mu\text{g/kg}$ for the IgM and IgG responses to SRBC. The no effect level was 0.056 $\mu\text{g/kg}$ for the immunological studies. Host resistance studies using two bacteria, (Listeria monocytogenes and Streptococcus pneumoniae), one virus (Herpes type 2), on protozoan (Plasmodium berghei) and one tumor model (B16F10 melanoma). The two models which showed increased resistance when the mice were exposed to HCDD were Listeria monocytogenes and Herpes virus type 2. A decreased resistance to Streptococcus pneumoniae occurred in mice exposed to the dioxins. The low doses of the dioxins decreased resistance to B16F10 melanoma, while the high doses demonstrated protection.

4,4'-Thiobis(6-t-butyl-m-cresol) (TBBC) was the last chemical to be investigated for immunotoxicity during the remainder of the contract period. TBBC was selected based on data from the NTP bioassay showing splenic atrophy and decreases in peripheral blood leucocytes. TBBC is an antioxidant widely used in the rubber and plastics industry. The doses for TBBC were set at 10, 100, and 200 mg/kg based on extrapolation from the NTP Bioassay feeding studies and from the pharmacokinetic studies of Birnbaum et al. (Drug Metab. Disp. 11:537-543, 1983). TBBC administered daily by gavage in corn oil for 14 consecutive days at doses of 10, 100 or 200 mg/kg produced no overt toxicity. The major target organ appears to be the liver, as seen in the histopathological examinations,

serum chemistries, and microsomal enzyme activities. The spleen of the exposed mice was slightly increased in weight and cellularity, but showed no histopathology. TBBC exposure in mice resulted in several effects on the immune system. TBBC caused a decrease in the number of splenic T cells, a decrease in IgM and IgG response to sheep erythrocytes, a decreased mixed lymphocyte response, an increase in number of peritoneal and liver macrophages (Kupffer cells), and an increase in serum complement and NK cell activity. All of the TBBC effects were dose dependent and the lowest no effect level was 10 mg/kg. TBBC caused an increased resistance in two of the six host resistance models, a decreased resistance in three models, and no change in resistance in one model. The three models whose host resistance was unaffected by TBBC were Herpes virus type 2, Listeria monocytogenes and Plasmodium berghei. The two models which showed increased resistance in mice exposed to TBBC were Streptococcus pneumoniae and B16F10 melanoma and the model where a decrease in resistance was observed was the PYB6 tumor. Although no relationship between deficit of a single immune parameter and decreased host resistance has been found, if any of the immune assays in the NTP panel are altered, resistance to one or more of the bacterial, viral or tumor challenge models is altered.

This contract expired on February 15, 1985.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A correlation has been clearly established between the administration of chemical immunosuppressants and an increased incidence of infectious diseases and neoplasia. The evidence for increased bacterial, viral, fungal and parasitic diseases in patients on chronic immunosuppressive chemicals has been well documented by Allen (Infection complicating neoplastic disease and cytotoxic therapy. In Infection and the Compromised Host, 1976). Likewise, McKhann (Transplantation 8:209, 1971) observed that the incidence of cancer in renal transplant recipients on prolonged immunosuppressive chemotherapy was 4.6-6.1 times higher than in the general population.

Studies in laboratory animals also have supported these clinical observations and demonstrated an enhanced incidence of UV-induced or benzopyrene-induced cancer in mice treated with immunosuppressive agents. The mechanisms and relationship between altered host resistance and immune dysfunction is complex, poorly defined and of extreme importance. Chemicals of environmental concern have been recently shown to induce immunosuppression as evidenced by depressed antibody mediated immunity, cell-mediated immunity or M ϕ dysfunction in rodents following sublethal exposure. Some of the chemicals which induce immunologic effects in rodents include 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated biphenyls, polybrominated biphenyls, gallic acid, DES, BP, hexachlorobenzene, pentachlorophenol, certain organo and heavy metals. Some studies have indicated that exposure to certain chemicals can alter resistance to bacteria, viruses, parasites and transplantable tumor cells. Of major concern is the correlation of these immunologic findings with altered host susceptibility and the extrapolation of these chemically-induced immunobiologic effects to humans.

RESEARCH TRIANGLE INSTITUTE - Research Triangle Park, NC 27709
(N01-ES-1-5007)

TITLE: "Pharmacokinetics of Xenobiotics"

CONTRACTOR'S PROJECT DIRECTOR: A Robert Jeffcoat, Ph.D.

PROJECT OFFICER (NIEHS): H. B. Matthews, Ph.D.

DATA CONTRACT INITIATED: July 15, 1981

CURRENT ANNUAL LEVEL: \$250,388

PROJECT DESCRIPTION

OBJECTIVES: The object of this contract is to provide information on the metabolism, distribution and excretion of selected xenobiotics which are of particular interest to the National Toxicology Program or intramural scientists at the NIEHS. These studies are designed to provide a better understanding of those factors which determine the rates of absorption, distribution and excretion of xenobiotics and to provide the data necessary to an estimation of the biological half-lives, times-to-steady-state and possible chronic toxicity of the compounds studied.

METHODS EMPLOYED: These studies will be conducted in intact animals and will utilize ^{14}C -labeled compounds or established analytical techniques to determine the degree of absorption, major tissue depots, clearance rates, degree of metabolism, and rates and routes of excretion. To achieve this a number of animals will be treated similarly, sacrificed in a serial manner and the major tissues and daily excreta of each animal will be sampled to determine the content of the compounds of interest and metabolites. The relative amounts of parent compound and metabolites will be determined at selected time points by extraction with organic solvents and various types of chromatographic analysis.

MAJOR FINDINGS AND PROPOSED COURSE:

1. The disposition of [^{14}C]-hexachloro-1,3-butadiene ([^{14}C]HCBd) was studied in 88-103 day old male Fischer 344 rats and 89-93 day old male B6C3F₁ mice. In rats, 47-63% of oral and intravenous doses up to 34 mg/kg dose was excreted in feces and 15-26% in urine in 3 days. Only small amounts of the dose were excreted in urine as the parent xenobiotic. After an oral dose of 166 mg/kg of [^{14}C]HCBd, however, only 5-20% of the dose was excreted in feces and 5-6% in urine during the same period. Large amounts of ^{14}C were present in the gastrointestinal tract contents of these animals after 72 h (ca 10% in the stomach, 34% in the cecum, 7% in the intestines). Approximately 10% of the dose was found in rat adipose 72 h after either intravenous or oral doses of HCBd, decreasing to 0.3-0.4% of the dose by 240 h. No mortality was observed in rats for doses up to 166 mg/kg.

Following oral (gavage) administration of [^{14}C]HCBd to mice at 2.0, 24, and 50 mg/kg and IV administration at 19 mg/kg, about half of the dose was excreted in feces in 2 days. After 24 and 50 mg/kg oral doses, urinary excretion (20 and 5% of the dose) was decreased as compared to the urinary

excretion following the 2.0 mg/kg oral dose (31%) or the IV dose (28%). Decreased food and water consumption was observed concomitant to this decrease in excretion rate. Toxicity of HCB_D following 27 and 110 mg/kg oral doses was significantly reduced when the hepatic glutathione levels in the mice were depleted with diethyl maleate pretreatment, suggesting that glutathione plays a major role in HCB_D metabolism and toxicity. Toxicity was not affected by depleting gut flora with antibiotic treatment prior to oral administration of HCB_D (27 and 110 mg/kg), suggesting that deconjugation by gut flora is not a major factor in the toxicity of HCB_D in mice. This study has been completed and is being prepared for publication.

2. The biocide, -bromo- -nitrostyrene, is being studied in the rat. The dermal absorption and disposition of both the phenyl ring (³H labeled) and side chain (¹⁴C labeled) portions of the molecule are being investigated. Effects of dose, route of exposure and position of the radiolabel on the rate and types of metabolism are being investigated to provide data for the design and interpretation of the chronic bioassay.
3. The comparative metabolism of the isomeric chlorohydrins 2-chloro-1-propanol and 1-chloro-2-propanol is under study in the rat. These compounds, which are important chemical intermediates, are known to undergo facile elimination of chloride in the presence of a suitable base. The fate of the individual compounds is being investigated because these two compounds are normally used as a mixture and there is some evidence that significant differences in metabolism may cause one compound to account for most of the toxicity of the mixture.
4. The dermal and oral absorption and the disposition of 4,5-dihydroxy-1,3-bis(hydroxymethyl)-2-imidazolidinone (DMDHEU) is being studied in the rat. DMDHEU, which is unstable in pure form, is being synthesized and administered as the reaction product of formaldehyde and 4,5-dihydroxy-2-imidazolidinone (DHEU). Effects of dose and route of exposure are being investigated to determine the design of the chronic bioassay. Results of these studies indicate that DMDHEU is poorly absorbed through the skin and it may not be possible to conduct a meaningful chronic study using dermal exposure. Oral doses may also be poorly absorbed and are very rapidly excreted in feces. DMDHEU has a whole body half-life of only a few hours.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is the goal of much biomedical research, the NTP, and the NIEHS to determine the significance of human exposure to a variety of toxic xenobiotics. A finite amount of data on the metabolism and disposition of toxic xenobiotics is essential to the proper design of chronic studies of such compounds. Furthermore, data obtained from carefully planned and executed studies of the metabolism and disposition of toxic xenobiotics can be used to more accurately relate laboratory observations to man. It is the role of this contract to provide disposition and kinetic data to complement studies of toxic xenobiotics which will be done under the NTP or in the NIEHS Intramural Program.

SOUTHERN RESEARCH INSTITUTE - Birmingham, Alabama 30332
(N01-ES-1-5008)

TITLE: "Pharmacokinetics of Xenobiotics"

CONTRACTOR'S PROJECT DIRECTOR: Donald L. Hill, Ph.D.

PROJECT OFFICER (NIEHS): H. B. Matthews, Ph.D.

DATA CONTRACT INITIATED: July 15, 1981

CURRENT ANNUAL LEVEL: \$239,424

PROJECT DESCRIPTION

OBJECTIVES: The object of this contract is to provide information on the metabolism, distribution and excretion of selected xenobiotics which are of particular interest to the National Toxicology Program or intramural scientists at the NIEHS. These studies are designed to provide a better understanding of those factors which determine the rates of absorption, distribution and excretion of xenobiotics and to provide the data necessary to an estimation of the biological half-lives, times-to-steady-state and possible chronic toxicity of the compounds studied.

METHODS EMPLOYED: These studies will be conducted in intact animals and will utilize ^{14}C -labeled compounds or established analytical techniques to determine the degree of absorption, major tissue depots, clearance rates, degree of metabolism, and rates and routes of excretion. To achieve this a number of animals will be treated similarly, sacrificed in a serial manner and the major tissues and daily excreta of each animal will be sampled to determine the content of the compounds of interest and metabolites. The relative amounts of parent compound and metabolites will be determined at selected time points by extraction with organic solvents and various types of chromatographic analysis.

MAJOR FINDINGS AND PROPOSED COURSE:

1. The fate of 9-aminoacridine, a broad spectrum topical antiseptic, has been studied in rats. Dermal absorption was studied in rats and monkeys. 9-Aminoacridine was poorly absorbed from the skin of both species. However, 9-aminoacridine was readily absorbed from the gastrointestinal tract of rats, readily metabolized and rapidly excreted. The metabolites of 9-aminoacridine are excreted primarily in bile and ultimately in feces. The remainder of the dose is excreted in urine. The major metabolite of 9-aminoacridine was isolated from urine and bile and identified as a glucuronide of the hydroxylated parent compound. Dermal absorption of 9-aminoacridine could not be demonstrated conclusively. Therefore, it may not be possible to do a meaningful toxicity study for this compound using the skin paint method of exposure.
2. Decabromodiphenyloxide is a major halogenated aromatic hydrocarbon fire retardant. Due to its complete halogenation it is poorly soluble in all solvents and therefore very poorly absorbed. A systematic study to determine the effect of dose on gastrointestinal absorption revealed that less

than 5% of a dose of this compound is absorbed from the gastrointestinal tract when administered at a dose range from 250 to 50,000 ppm in the diet. This study did confirm a trace of absorption at all doses and confirmed that the material absorbed was the parent compound. Studies of decabromodiphenyl oxide injected intravenously indicate that this completely halogenated aromatic may be metabolized and excreted in bile. Results of these studies are being used to interpret the results of a two-year study of the chronic toxicity and carcinogenicity of decabromodiphenyl oxide.

3. The disposition and metabolism of 2-hydroxy-4-methoxybenzophenone (HMB), a sunscreen agent, is being investigated in rats following oral and dermal administration. Results of these studies indicate that this compound is readily absorbed from the gastrointestinal tract with no apparent saturation across a dose range of 3 to 3,000 mg/kg. HMB is readily metabolized and excreted in urine. HMB does not appear to persist in tissues even after high doses. Studies of dermal absorption indicate that HMB is absorbed at the rate of approximately 10 to 20% of the dose. Disposition and excretion following dermal absorption of HMB appears to be similar to that observed following oral administration. Results of these studies will be used to plan a study of HMB following administration by skin paint and possibly in the diet as well.
4. N-(3-chloroallyl)hexaminium chloride (CHC) is widely used as a biocide in paint and in numerous cosmetics. This chemical has been nominated for study in the NTP bioassay. The disposition and metabolism of this chemical is currently being studied to provide data to be used in the design of chronic toxicity and carcinogenicity studies. Results of the disposition studies indicate that CHC administered orally is readily absorbed from the gastrointestinal tract, readily metabolized and excreted as CO₂ in exhaled air and as polar metabolites in urine and feces. There was little effect of dose on disposition in the dose range studied (2-50 mg/kg). The biocidal activity of CHC has been attributed to the slow release of formaldehyde. A special effort to detect formaldehyde in exhaled air provided negative results; therefore, if formaldehyde is formed in the intact animals it is apparently degraded to CO₂ prior to elimination.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is the goal of much biomedical research, the NTP, and the NIEHS to determine the significance of human exposure to a variety of toxic xenobiotics. A finite amount of data on the metabolism and disposition of toxic xenobiotics is essential to the proper design of chronic studies on such compounds. Furthermore, data obtained from carefully planned and executed studies of the metabolism and disposition of toxic xenobiotics can be used to more accurately relate laboratory observations to man. It is the role of this contract to provide disposition and kinetic data to complement studies of toxic xenobiotics which will be done under the NTP or in the NIEHS Intramural Program.

PUBLICATIONS

El Dareer, S. M., Kalin, J. A., Tillery, K. F. and Hill, D. L.: Disposition of 2-mercaptobenzimidazole in rats dosed orally or intravenously. J. Toxicol. Environ. Health 14: 595-604, 1984.

El Dareen, S. M., Kalin, J. A., Tillery, K. F. and Hill, D. L.: Disposition of 9-aminoacridine in rats dose orally or intravenously and in monkeys dosed topically. J. Toxicol. Environ. Health. In Press, 1985.

LOVELACE INHALATION TOXICOLOGY RESEARCH INSTITUTE
Albuquerque, NM
(Interagency Agreement with the Department of Energy)
(22Y01-ES-20092)

TITLE: "Disposition of Inhaled Xenobiotics"

CONTRACTOR'S PROJECT DIRECTOR: Rogene Henderson, Ph.D.

PROJECT OFFICER (NIEHS): L. S. Birnbaum, Ph.D., Research Microbiologist,
Chemical Disposition, Systemic Toxicology Branch

DATA CONTRACT INITIATED: September 30, 1982

CURRENT ANNUAL LEVEL: \$734,334

PROJECT DESCRIPTION

OBJECTIVES: The objective of this contract is to provide information on the metabolism, distribution and excretion of selected volatile xenobiotics which are of particular interest to the National Toxicology Program or scientists in the intramural program at the NIEHS. These studies are designed to provide a better understanding of those factors which determine the rates of absorption, distribution and excretion of xenobiotics and to provide the data necessary to an estimation of the biological half-lives, times-to-steady-state and possible chronic toxicity of the compounds studied.

METHODS EMPLOYED: These studies will be conducted in intact animals and will utilize ^3H or ^{14}C labeled compounds or established analytical techniques to determine the degree of absorption, major tissue depots, clearance rates, degree of metabolism and rates and routes of excretion. To achieve this, a number of animals will be treated similarly, sacrificed in a serial manner, and the major tissues and daily excreta of each animal will be sampled to determine the content of the compounds of interest. The relative amounts of parent compound and metabolites will be determined at selected time points by extraction with organic solvents and various types of chromatographic analysis. For compounds of great toxicological interest, metabolites will be determined and DNA binding assessed.

MAJOR FINDINGS: 1) The disposition of ^{14}C -2,3-dichloropropene (DCP), a soil fumigant, was examined after inhalation exposure in Fischer 344 rats. Absorption, metabolism and excretion were linear over a dose range from 17 to 1700 nmole DCP/liter for 6 hours. Steady state was reached after 4 hours. DCP was widely distributed in the tissues and rapidly eliminated with half-lives ranging from 3 to 11 hrs. Most of the radioactivity in the tissues was metabolite(s) of DCP as was that in excreta. Similar excretion patterns were seen after inhalation exposure as after oral and ip administration. Urine was the major route of excretion (70%), followed by feces (20%), and CO_2 (8%). Only 2% of the observed radioactivity remains in the body 3 days after treatment.

2) The disposition of ^{14}C -methyl bromide (MB), a major industrial chemical, was studied after inhalation exposure. While metabolism and excretion were linear

based upon the absorbed dose from 50 to 10,400 nmoles/liter for 6 hrs, absorption decreased at the higher exposure concentrations. Thus rats absorbed the same amount of MB when breathing 5700 nmoles MB/liter as when exposed to 10,400 nmoles/l. Half of the absorbed MB was eliminated as $^{14}\text{CO}_2$, 20% appeared in urine, 2% in feces, 4% was exhaled as unchanged MB, and approximately 25% remained in the rats. This residual radioactivity may have been incorporated into the one-carbon pool for intermediary metabolism. The excretion patterns of CO_2 and of radioactivity in urine were similar after ip and inhalation exposure, but more unaltered MB was exhaled after ip treatment. Oral treatment resulted in more liver metabolism and a greater amount of renal rather than lung clearance.

Steady state conditions in the blood were not reached even after 6 hours of exposure. The MB-derived radioactivity was widely distributed and cleared from the tissues with half-lives ranging from 2-3 hours, except in the liver where radioactivity tended to persist. Excretion in urine had a half-life of 10 hours. Results indicate that MB is rapidly metabolized and excreted as CO_2 or in urine.

Mass-balance studies revealed that a standard CO_2 trapping agent, ethanolamine, was not adequate for metabolism studies and alternative methodology was developed.

3) Azodicarbonamide (ADA) is a thermoplastic blowing agent and dough conditioner. Rats were exposed nose-only to 1.5, 15, or 150 $\mu\text{C-ADA/l}$ air for 4 hours. Using plethysmograph units, it was determined that 15% of the inhaled aerosol was deposited in the rats. Blood levels continued to rise throughout the exposure. Excretion was primarily in the feces (32%), followed by urine (13%) and CO_2 (<2%). Rates and routes of excretion were independent of exposure concentrations. The pattern of excretion was similar to that observed after oral exposure. This indicates that much of an inhaled dose is actually swallowed. Intratracheal administration resulted in 90% of the dose being excreted in the urine.

ADA is rapidly metabolized to biurea. Essentially all the radioactivity in the urine and feces is biurea. Little ADA-derived radioactivity persists in the body 3 days after exposure.

4) The disposition of inhaled 1,3-butadiene (BD), a chemical used in the manufacture of polymers, is being studied in male Sprague Dawley rats and male B6C3F₁ mice to determine if species differences in disposition can explain species differences in the tumorigenic response to inhaled BD. Initial exposures of mice to three concentrations of $^{14}\text{C-BD}$ (14, 180, 1400 $\mu\text{g BD/L}$ air) for 6 hr indicated that rates and routes of excretion were not dose dependent over the range studied. Within 65 hrs after exposure, 50% of the absorbed radioactivity was excreted in urine and 7% in feces. Eleven percent was exhaled as CO_2 and 5% as unaltered BD. Seventy-eight percent of the urinary radioactivity was excreted with a half-life of 17 hr. This can be compared with the excretion pattern observed when the mice were given BD intraperitoneally. In the latter study, 75% was exhaled as BD, with only small amounts excreted by other routes. This evidently results from loss of BD from the blood during its first pass through the lungs.

Further methods development has led to techniques that will allow separation of BD from its volatile metabolites (one of which is butadiene monoxide) in blood, tissues and excreta. Rats and mice have now been exposed to a range of 3 BD air concentrations and the excretion of BD and its metabolites and the uptake into blood measured. These data are still undergoing analysis and should indicate the degree of metabolism of the inhaled BD. Some rodents were also exposed in plethysmograph units to allow determination of the percent of inhaled BD that was absorbed.

5) Benzene has been shown to increase the incidence of tumor formation in Sprague-Dawley rats treated by the oral or inhalation route and in F344 rats and B6C3F₁ mice treated orally. The distribution of benzene and its metabolites in selected tissues is being studied in these three animal strains following an oral or inhalation route of exposure. Animals will be exposed to ³H-benzene at 0.5 to 300 mg/kg/day (oral) or 1.0 to 300 ppm/6 hr/day (inhalation) for 5 days. Following the final exposure, animals will be sacrificed and benzene and its metabolites quantitated in urine, blood, feces, liver, lung, bone marrow, and Zymbals gland. Metabolites in oral cavity, stomach, skin around the mouth and skin on the back will be analyzed only at the low and high doses. Metabolites to be analyzed by HPLC include benzene, trihydroxy benzene, hydroquinone, catechol, phenol, muconic acid and water soluble conjugates.

In addition to the metabolite studies, the effects of inhaled benzene concentration and exposure time on the formation of chromosomal aberrations and sister chromatid exchanges in blood lymphocytes and liver are under way. Formation of micronuclei in bone marrow cells from the exposed rodents will be evaluated by NIEHS personnel. Covalent binding of benzene and/or its metabolites to DNA will also be measured.

To date, exposure of B6C3F₁ mice to 0.5, 15 and 300 mg/kg/day ³H-benzene by the oral route has been completed and tissue samples are currently being analyzed for unconjugated metabolites. At 15 mg/kg, the benzene was labeled with both ¹⁴C and ³H to determine whether an isotope effect would be seen. F344 rats have been exposed at 50 mg/kg/day and tissues analyzed by HPLC for unconjugated metabolites. To determine the actual exposure of the animal to a given dose of benzene requires that the amount of uptake be known. Therefore, we are investigating the gastrointestinal, pulmonary and skin uptake of ¹⁴C-benzene. Gastrointestinal uptake studies are nearing completion and indicate nearly 100% uptake of orally administered benzene by the GI tract in all three animal strains. At 300 mg/kg, ~ 60% of the administered benzene is exhaled with the remainder excreted in the urine. However, at a low dose of 0.5 mg/kg, almost all of the ¹⁴C-benzene-equivalents are excreted in the urine, indicating that metabolism is not saturated at this lower dose.

Exposure of animals to ³H-benzene by the inhalation route will begin in June 1985 and should be completed by December 1985. Pulmonary and skin uptake of benzene will also be determined during this time.

PROPOSED COURSE:

1. Additional compounds will be studied as requested by NTP personnel.
¹⁴C-isoprene will be studied because of its relationship to butadiene.
¹⁴C-vinylidene fluoride will also be studied.

2. Because of the need for additional rodent information to better extrapolate risks to humans, this agreement was modified to allow extensive dosimetry studies of benzene exposure. Design of these studies has involved Dr. George Lucier of BRAP. In addition to disposition, DNA-binding and cytogenetic endpoints will also be studied.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is the goal of much biomedical research, the NTP, and the NIEHS to determine the significance of human exposure to a variety of toxic xenobiotics. A finite amount of data on the metabolism and disposition of toxic xenobiotics is essential to the proper design of chronic studies of such compounds. Furthermore, data obtained from carefully planned and executed studies of the metabolism and disposition of toxic xenobiotics can be used to more accurately relate laboratory observations to man. It is the role of this contract to provide disposition and kinetic data which will complement studies of toxic xenobiotics under the NTP or in the NIEHS Intramural Program.

PUBLICATIONS

Medinsky, M. A., Bond, J. A., Dutcher, J. S., and Birnbaum, L. S.: Disposition of ^{14}C -methyl bromide in Fischer-344 rats after oral or intraperitoneal administration. Toxicology 32:187-196, 1984.

Medinsky, M. A., Bond, J. A., Dutcher, J. C., and Birnbaum, L. S.: Disposition of ^{14}C -2,3-dichloropropene in Fischer-344 rats after oral or intraperitoneal administration. Tox. Letts. 23:119-125, 1984.

Bond, J. A., Medinsky, M. A., Dutcher, J. S., Henderson, R. F., Cheng, Y. S., Mewhinney, J. A., and Birnbaum, L. S.: Disposition and metabolism of ^{14}C -2,3-dichloropropene in rats after inhalation. Toxicol. Appl. Pharmacol. 78:47-54, 1985.

Dutcher, J. S., Medinsky, M. A., Bond, J. A., Cheng, Y. S., Snipes, M. B., Henderson, R. F., and Birnbaum, L. S.: Effect of vapor concentration on the disposition of inhaled 2,3-dichloropropene in Fischer-344 rats. Fund. Appl. Toxicol. In press.

Bond, J. A., Dutcher, J. S., Medinsky, M. A., Henderson, R. F., Cheng, Y. S., Mewhinney, J. A. and Birnbaum, L. S.: Disposition and metabolism of ^{14}C -methyl bromide in rats after inhalation. Toxicol. Appl. Pharmacol. 78:259-267, 1985.

Medinsky, M. A., Dutcher, J. S., Bond, J. A., Henderson, R. F., Mauderly, J. L., Snipes, M. B., Mewhinney, J. A., Cheng, Y. S. and Birnbaum, L. S.: Uptake and excretion of ^{14}C -methyl bromide as influenced by exposure concentration. Toxicol. Appl. Pharmacol. 78:215-225, 1985.

ENVIRONMENTAL HEALTH RESEARCH AND TESTING, INC.
Lexington, Kentucky 40503
(NIH-N01-ES-2-5013)

TITLE: Fertility Assessment by Continuous Breeding

CONTRACTOR'S PROJECT DIRECTOR: D.K. Gulati, Ph.D.

PROJECT OFFICER (NIEHS): James C. Lamb, IV, Ph.D.
Head, Fertility and Reproduction Group, STB

DATE CONTRACT INITIATED: January 29, 1982

CURRENT ANNUAL LEVEL: \$490,000

PROJECT DESCRIPTION

OBJECTIVES: This project is designed to evaluate a new reproductive toxicology testing system.

METHODS EMPLOYED: This reproductive toxicology testing system employs an extended chemical exposure and a protocol which includes the mating of continuously-exposed male and female mice. Mating pairs will be housed together for 100 days and offspring will be counted to determine an index of cumulative fertility. The system allows for testing offspring collected between 100 and 120 days, if the parental generation has not been adversely affected by the chemical exposure. The test system may also be used to identify the affected sex or study various target organ response with a special necropsy which focuses on reproductive target organs. The special organ response studies may include sperm concentration, sperm morphology, vaginal cytology and histopathology.

MAJOR FINDINGS AND PROPOSED COURSE: This contract was awarded in the second quarter of FY 1982. Chemical testing has been completed for 24 chemicals; reports are being prepared. This phase of testing is designed to evaluate the testing system's utility. This contract has provided new methods for evaluating reproductive toxicity and important data on the effects of certain chemicals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study is an essential element in the National Toxicology Program's initiative in reproductive toxicology test development and validation. This is a new testing protocol which will be compared to other, more expensive testing systems and should lead us to new and improved reproductive toxicity testing systems.

PUBLICATIONS

Lamb, J.C., IV, Gulati, D.K., Russel, V.S., Hommel, L. and Sabharwal, P.S.: Reproductive toxicity of ethylene glycol monoethyl ether tested by continuous breeding of CD-1 mice. Environ. Hlth. Perspect., 57: 85-90, 1984.

Lamb, J.C., IV, Jameson, C.W., Choudhury, H. and Gulati, D.K.: Fertility assessment by continuous breeding: Evaluation of diethylstilbestrol and a comparison of results from two laboratories. J. Amer. Coll. Toxicol., 4: 173-184, 1985.

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- Gulati, D.K., Russell, V.S., Hommel, L., Poonacha, K.B., Sabharwal, P.S. and Lamb, J.C. IV: Ethylene glycol: Fertility assessment in CD-1 mice when administered in drinking water. National Toxicology Program, Technical Report NTP-84-155, 1984, 234 pp.
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- Gulati, D.K., Choudhury, H., Chambers, R., Poonacha, K.B., Sabharwal, P.S. and Lamb, J.C.: Diethylstilbestrol: Fertility assessment in CD-1 mice when administered in feed. National Toxicology Program, NTP-85-016, 1985, 225 pp, NTIS No. PB85-167674/as.
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- Gulati, D.K., Chambers, R., Shaver, S., Sabharwal, P.S., and Lamb, J.C., IV: Theophylline: Reproduction and fertility assessment in CD-1 mice when administered in drinking water. National Toxicology Program, Technical Report NTP-85-096, 1985, 330 pp.
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- Gulati, D.K., Russell, V.S., Hommel, L., Poonacha, K.B., Sabharwal, P.S., and Lamb, J.C., IV: Caffeine: Reproduction and fertility assessment in CD-1 mice when administered in drinking water. National Toxicology Program, Technical Report NTP-85-097, 1985, 243 pp.

RESEARCH TRIANGLE INSTITUTE
Research Triangle Park, North Carolina 27709
(NIH-N01-ES-2-5014)

TITLE: Fertility Assessment by Continuous Breeding

CONTRACTOR'S PROJECT DIRECTOR: J.R. Reel, Ph.D.

PROJECT OFFICER (NIEHS): James C. Lamb, IV, Ph.D.
Head, Fertility and Reproduction Group, STB

DATE CONTRACT INITIATED: January 27, 1982

CURRENT ANNUAL LEVEL: \$359,000

PROJECT DESCRIPTION

OBJECTIVES: This project is designed to evaluate a new reproductive toxicology testing system.

METHODS EMPLOYED: This reproductive toxicology testing system employs an extended chemical exposure and a protocol which includes the mating of continuously-exposed male and female mice. Mating pairs will be housed together for 100 days and offspring will be counted to determine an index of cumulative fertility. The system allows for testing offspring collected between 100 and 120 days, if the parental generation has not been adversely affected by the chemical exposure. The test system may also be used to identify the affected sex or study various target organ response with a special necropsy which focuses on reproductive target organs. The special organ response studies may include sperm concentration, sperm morphology, vaginal cytology and histopathology.

MAJOR FINDINGS AND PROPOSED COURSE: This contract was awarded in the second quarter of FY 1982. Chemical testing has been completed for sixteen chemicals and reports are being prepared. This phase of testing is designed to evaluate the testing system's utility. This contract has provided new methods for evaluating reproductive toxicity and important data on the effects of certain chemicals.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study is an essential element in the National Toxicology Program's initiative in reproductive toxicology test development and validation. This is a new testing protocol which will be compared to other, more expensive testing systems and should lead us to new and improved reproductive toxicity testing systems.

PUBLICATIONS

Reel, J.R., Lawton, H.D., Wolkowski-Tyl, R., Davis, G.W. and Lamb, J.C., IV: Evaluation of a new reproductive toxicology protocol using diethylstilbestrol (DES) as a positive control compound. J. Amer. Coll. Toxicol., in press.

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Reel, J.R., Wolkowski-Tyl, R., Lawton, A.D. and Lamb, J.C.: Bisphenol A: reproduction and fertility assessment in CD-1 mice when administered via subcutaneous silastic implants. National Toxicology Program, Technical Report NTP-84-015, 1984, 182 pp.

Reel, J.R., Wolkowski-Tyl, R., Lawton, A.D. and Lamb, J.C., IV: Sulfamethazine: Reproductive and fertility assessment in CD-1 mice when administered via the diet. National Toxicology Program, Technical Report NTP-84-092, 1984, 218 pp.

Reel, J.R., Wolkowski-Tyl, R., Lawton, A.D. and Lamb, J.C., IV: Diethylhexyl phthalate (DEHP): Reproduction and fertility assessment in CD-1 mice when administered in the feed. National Toxicology Program, Technical Report NTP-84-079, 1984, 217 pp.

Reel, J.R., Wolkowski-Tyl, R., Lawton, A.D. and Lamb, J.C., IV: Caffeine: Reproductive and fertility assessment in CD-1 mice when administered in the drinking water. National Toxicology Program, Technical Report NTP-84-158, 1984, 203 pp.

Reel, J.R., Wolkowski-Tyl, R., Lawton, A.D. and Lamb, J.C., IV: Lead acetate (PbAc): Reproduction and fertility assessment in CD-1 mice when administered in the drinking water. National Toxicology Program, Technical Report NTP-84-108, 1984, 208 pp.

Reel, J.R., Wolkowski-Tyl, R., Lawton, A.D. and Lamb, J.C., IV: Methyl salicylate: Reproduction and fertility assessment in CD-1 mice when administered by gavage. National Toxicology Program, NTP-84-156, 1984, 207 pp.

Reel, J.R., Wolkowski-Tyl, R., Lawton, A.D. and Lamb, J.C.: Dibromochloropropane: Reproduction and fertility assessment in CD-1 mice when administered by gavage. National Toxicology Program, Technical Report NTP-84-263, 1984, 202 pp, NTIS No. PB85-118644.

Reel, J.R., Lawton, A.D. and Lamb, J.C., IV: Diethylphthalate: Reproduction and fertility assessment in CD-1 mice when administered in the feed. National Toxicology Program, Technical Report NTP-84-262, 1984, 187 pp, NTIS No. PB85-118636.

Reel, J.R., Lawton, A.D., and Lamb, J.C., IV: Triethylene glycol: Reproduction and fertility assessment in CD-1 mice when administered in the drinking water. National Toxicology Program, Technical Report NTP-84-407, 1984, 187 pp, NTIS No. PB85-137073.

Reel, J.R., Lawton, A.D., and Lamb, J.C., IV: Diethylene glycol monoethyl ether: Reproduction and fertility assessment in CD-1 mice when administered in the drinking water. National Toxicology Program, Technical Report NTP-84-406, 1984, 179 pp, NTIS No. PB85-137123.

Reel, J.R., Lawton, A.D., and Lamb, J.C., IV: Ethylene glycol monophenyl ether: Reproductive and fertility assessment in CD-1 mice when administered in the feed. National Toxicology Program, Technical Report NTP-84-410, 1984, 244 pp, NTIS PB85-146140/AS.

Reel, J.R., Lawton, A.D., and Lamb, J.C., IV: Di-n-butyl phthalate: Reproductive and fertility assessment in CD-1 mice when administered in the feed. National Toxicology Program, Technical Report NTP-84-411, 1984, 197 pp, NTIS PB85-144798.

Reel, J.R., Lawton, A.D., George, J.D., and Lamb, J.C., IV: Triethylene glycol dimethyl ether: Reproduction and fertility assessment in CD-1 mice when administered in the drinking water. National Toxicology Program, Technical Report NTP-85-007, 1985, 194 pp, PB85-5150456/AS.

Reel, J.R., Lawton, D.A., and Lamb, J.C., IV: Acetaminophen: Reproduction and fertility assessment in CD-1 mice when administered in water. National Toxicology Program, Technical Report NTP-85-095, 1985, 207 pp.

ENVIRONMENTAL HEALTH RESEARCH AND TESTING, INC.
Lexington, Kentucky 40503
(NIH-N01-ES-3-5026)

TITLE: Sperm Morphology and Vaginal Cytology Evaluation

CONTRACTOR'S PROJECT DIRECTOR: D.K. Gulati, Ph.D.

PROJECT OFFICER (NIEHS): James C. Lamb, IV, Ph.D.
Head, Fertility and Reproduction Group, STB

DATE CONTRACT INITIATED: May 1, 1983

CURRENT ANNUAL LEVEL: \$89,000

PROJECT DESCRIPTION

OBJECTIVES: This contract was designed to supply a method for screening chemicals for reproductive toxicity. It standardizes and centralizes the collection of data collected from studies run in the numerous testing laboratories in the Bioassay Program. The system allows for the collection of reproductive toxicity data without purchasing additional animals, test chemical or animal care expenses. This arrangement also facilitates interstudy comparisons of reproductive toxicity.

METHODS EMPLOYED: Approximately twenty new chemicals per year begin testing in the Bioassay Program. The special reproductive toxicity testing screens used include sperm concentration, motility and morphology in male rats and mice and vaginal cyclicity in female rats and mice. The NTP bioassay testing laboratories collect the specimens, prepare the slides and ship them to this NTP-designated laboratory. EHRT is responsible for providing technical direction, evaluation, quality assurance, data summary and reports and slide inventory and storage.

MAJOR FINDINGS AND PROPOSED COURSE: Technical direction has been given by EHRT to bioassay laboratories before any slides have been collected. This should help assure uniformity in data collection and slide preparation. Slides will be sent to EHRT over the remainder of the contract; the protocol may be modified as the testing continues.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This reproductive toxicity testing system is an important component of the National Toxicology program's Reproductive and Developmental Toxicology Program. This system represents an effort to effectively use animals which are already on test in the Bioassay Program and thereby eliminate redundant animal dosing, care and necropsy and enhance our ability to identify reproductive toxicants. EHRT is responsible for assisting in the coordination of this extensive effort and assuring high quality specimen collection and data analyses. These studies serve as a unique and cost-effective prescreening system for reproductive toxicology.

UNIVERSITY OF ARIZONA - Tucson, Arizona 85724
(NO1-ES-3-5031)

TITLE: "Studies of Chemical Disposition in Mammals"

CONTRACTOR'S PROJECT DIRECTOR: I. Glenn Sipes, Ph.D.

PROJECT OFFICER (NIEHS): H. B. Matthews, Ph.D., Research Chemist, TRTP

DATE CONTRACT INITIATED: September 15, 1983

CURRENT ANNUAL LEVEL: \$258,231

PROJECT DESCRIPTION

OBJECTIVES: The objective of this contract is to provide research into the mechanisms of chemical disposition by higher animals and the relation of chemical disposition to chemical toxicity. These studies are designed to provide greater insight into those factors which determine rates of absorption, metabolism, tissue distribution and excretion of selected chemicals and how these parameters of chemical disposition are modulated by dose, route of exposure or other factors. The purpose of this work is to provide data which will permit more accurate prediction of bioaccumulation and chronic toxicity which are likely to occur as a result of chronic exposure or to provide insight into incidences of chronic toxicity observed in the course of chronic exposure.

METHODS EMPLOYED: These studies will be conducted in intact animals and/or isolated cells or enzymes and will utilize ¹⁴C-labeled compounds or established analytical techniques to determine the degree of absorption, major tissue depots, clearance rates, degree of metabolism and rates and routes of excretion. To achieve this, a number of animals will be treated similarly, sacrificed in a serial manner and the major tissues and daily excreta of each animal will be sampled to determine the content of the compounds of interest and metabolites. The relative amounts of parent compound and metabolites will be determined at selected time points by extraction with organic solvents and various types of chromatographic analysis. Isolated cells or enzymes may be used to study in vitro metabolism and/or toxicity.

MAJOR FINDINGS AND PROPOSED COURSE:

1. A continuing effort to quantitate the interactive toxicity induced by mixtures of chemicals has concentrated on hepatotoxicity induced by mixtures of chlorinated compounds. Pretreatment of rats with trichloroethylene or chloroform dramatically potentiated the hepatotoxicity of carbon tetrachloride. Similarly, concomitant administration of these two agents also resulted in marked potentiation of carbon tetrachloride induced liver injury. Chlorobenzene produced a marginal interactive effect (slightly enhanced CCl₄ toxicity), while concomitant administration of CCl₄ and 1,2-dichlorobenzene resulted in a very dramatic CCl₄-induced antagonism of 1,2-dichlorobenzene hepatotoxicity. Particular emphasis is being placed on understanding the mechanism(s) of these interactive events. The studies follow two paths for the potentiation of CCl₄-hepatotoxicity by trichloroethylene and chloroform - an alteration in the biotransformation of CCl₄ and an enhanced susceptibility of hepatocellular membranes of CCl₄-induced lipoperoxidation.

Additional information is being developed on the degree of potentiation/antagonism of chlorobenzene and 1,2-dichlorobenzene induced hepatotoxicity. Early results indicate that the mechanism involved may be related to hepatic glutathione depletion by the interactive chemical. However, lipoperoxidation may also be involved in interactive toxicity and this mechanism is being monitored closely as well. Studies are continuing with chlorobenzene, CCl₄ and trichloroethylene alone and in combination to determine if in vitro systems can be used to predict interactive hepatotoxic events. At present, studies are being conducted to determine if biochemical markers can be used as sensitive indices of toxicity. These include protein, lipid and glycogen synthesis, response to critical hormones (i.e. glucagon, insulin, steroids) and calcium homeostasis. Such studies will reveal alterations in a biological response and allow us to determine if this response or the degree of this response can predict a toxic response (cell death, calcium accumulation, potassium or enzyme leakage).

2. 4-Vinylcyclohexene (4-VCM) is an intermediate in the production of 4-epoxyethyl-1,2-epoxycyclohexane, a reactive diluent in the production of epoxy resins. In the production of synthetic rubber, 4-VCM is released into the air during the curing process for tire manufacture. In a 2-year study performed in B6C3F₁ mice and F344/N rats, 200 and 400 mg/kg of 4-VCM was administered orally 5 days a week. Female B6C3F₁ mice developed a significant number of ovarian neoplasms when compared to vehicle controls. Female F344/N rats did not. Current work is testing the hypothesis that the differences observed in the incidence of ovarian neoplasms between B6C3F₁ mice and F344/N mice are related to differences in the disposition and/or metabolism of 4-VCM in the ovary. The nature of the radiolabel present in the ovaries will be determined (parent compound, metabolites, bound material). If bound material is present, it will be fractionated into protein and nucleic acids. The metabolism of 4-VCM by ovary tissue from each species is being studied in vitro.
3. p-Nitrobenzoyl chloride is used as an intermediate in organic synthesis and has been detected as an environmental contaminant in the Love Canal chemical dumpsite. This chemical and m-nitrobenzoyl chloride have been chosen for study by the NTP. p-Nitrobenzoyl chloride has been chosen as representative of the two for studies of stability, disposition, metabolism and covalent binding. This chemical is quite reactive, but sufficiently stable in corn oil to permit oral gavage. It is rapidly degraded and rapidly excreted in urine primarily as p-nitrobenzoic acid. Tissues of the stomach and upper small intestine show little bound radioactivity 4 hr post dosing indicating minimal binding of this acyl chloride with tissue nucleophiles. Work is continuing to determine the amount of parent compound absorbed. Results of these studies will be used to design toxicity studies for this compound.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is the goal of much biomedical research, the NTP, and the NIEHS to determine the significance of human exposure to a variety of toxic xenobiotics. A finite amount of data on the metabolism and disposition of toxic xenobiotics is essential to the proper design of chronic studies of such compounds. Furthermore, data obtained from carefully planned and executed studies of the metabolism and disposition of toxic xenobiotics can be used to more accurately relate laboratory observations to man. It is the role of this contract to provide disposition and kinetic data

to complement studies of toxic xenobiotics which will be done under the NTP or in the NIEHS Intramural Program.

PUBLICATIONS

Volp, R. F., Gross, J. F., Falcoz, C., Carter, D. E. and Sipes, I. G.: Disposition of 1,2,3-trichloropropane in the Fischer 344 rat: Conventional and physiological pharmacokinetics. Toxicol. Appl. Pharmacol. 74:8-7, 1984.

Schnellmann, R. G., Volp, R. F., Putnam, C. W. and Sipes, I. G.: The hydroxylation, dechlorination, and glucuronidation of 4,4'-dichlorobiphenyl(4-DCB) by human hepatic microsomes. Biochem. Pharm. 33: 3503-3509, 1984.

Schnellmann, R. G. and Sipes, I. G.: Significance of metabolism studies in toxicity testing and risk assessment. In: New Approaches in Toxicity Testing and their Application to Human Risk Assessment. A.P. Li (ed) Raven Press, pp. 119-129, 1985.

Webb, D. R. and Carter, D. E.: Improved direct hydride method for the analysis of total arsenic in biological samples. Analytical Toxicology. 8:118-123, 1984.

Webb, D. R., Sipes, I. G. and Carter, D. E.: Clearance and toxicity of gallium arsenide particulates in Fischer 344 rats. Toxicol. Appl. Pharmacol. 76: 96-104, 1984.

Miller, M. J., Sipes, I. G., Perry, D. and Carter D. E.: Pharmacokinetic of o-nitroanisole in Fischer-344 rats. Drug Metab. Dispos. In press, 1985.

Miller, M. J., Miller, M. S., Burks, T. F. and Sipes, I. G.: A simple sensitive method for detecting early peripheral nerve dysfunction in the rat following acrylamide treatment. Neurotoxicology. In press, 1985.

Connors, S., Meyer, F. J., Sundheimer, D. W., I. G. and Brendel, K.: An all glass apparatus for liver perfusion of lipophilic substrates. J. Pharmacol. Methods 13: 83-94, 1985.

Wiersma, D. A., Schnellmann, R. G. and Sipes, I. G.: Pathways of halogenated hydrocarbon metabolism. in Foreign Compound Metabolism, pp 53-64, Taylor and Frances Ltd., London, 1984.

MICROBIOLOGICAL ASSOCIATES
Bethesda, Maryland 20816
(NIH N01-ES-3-8045)

TITLE: Evaluation of Two In Vitro Teratogenesis Testing Systems

CONTRACTOR'S PROJECT DIRECTOR: L. Li Yang, Ph.D.

PROJECT OFFICER (NIEHS): James C. Lamb, IV, Ph.D.
Head, Fertility and Reproduction Group, STB

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$166,000

PROJECT DESCRIPTION

OBJECTIVES: The objective of the proposed research is to validate two in vitro cell culture systems for the detection of teratogens in two independent laboratories. The in vitro testing systems will allow the Toxicology Research and Testing Program (TRTP) to assign priorities to chemicals for conventional, whole animal teratology testing. Successful screening systems can increase the cost effectiveness of TRTP testing activities.

METHODS EMPLOYED: Two cell culture systems will be evaluated. The first system evaluates the ability of chemicals to inhibit ascites Mouse Ovarian Tumor (MOT) cell attachment to concanavalin A-coated disks (Braun et al., Proc. Natl. Acad. Sci. USA, 79:2056, 1982). The second system measures the growth inhibition potential of Human Embryonic Palatal Mesenchyme (HEPM) cells in culture (Pratt et al., Teratogen. Carcinogen. Mutagen., 1982). The HEPm cell system has been reported to be complimentary to the tumor cell attachment assay.

Both assays will be run simultaneously at each contract laboratory. Forty to 50 chemicals, known teratogens and non-teratogens, will be selected and tested in each system for validation purposes.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory has improved the assay systems by standardizing assay conditions and identified certain objective criteria for judging an assay as valid. Testing has been completed on about 15 chemicals and testing on all 45 chemicals should be completed by September 1986.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Successful validation of the screening systems could help us to (1) accelerate the rate at which chemicals are evaluated for teratogenic potential; (2) improve the breadth of data on potential teratogens; (3) improve the selection of chemicals for whole animal testing; and (4) decrease testing cost.

NORTHROP SERVICES, INC.
Research Triangle Park, North Carolina 27709
(NIH N01-ES-3-8046)

TITLE: Evaluation of Two In Vitro Teratogenesis Testing Systems

CONTRACTOR'S PROJECT DIRECTOR: Vernon E. Steele, Ph.D.

PROJECT OFFICER (NIEHS): James C. Lamb, IV, Ph.D.
Head, Fertility and Reproduction Group, STB

DATE CONTRACT INITIATED: September 30, 1983

CURRENT ANNUAL LEVEL: \$162,000

PROJECT DESCRIPTION

OBJECTIVES: The objective of the proposed research is to validate two in vitro cell culture systems for the detection of teratogens in two independent laboratories. The in vitro testing systems will allow the Toxicology Research and Testing Program (TRTP) to assign priorities to chemicals for conventional, whole animal teratology testing. Successful screening systems can increase the cost effectiveness of TRTP testing activities.

METHODS EMPLOYED: Two cell culture systems will be evaluated. The first system evaluates the ability of chemicals to inhibit ascites Mouse Ovarian Tumor (MOT) cell attachment to concanavalin A-coated disks (Braun et al., Proc. Natl. Acad. Sci. USA, 79:2056, 1982). The second system measures the growth inhibition potential of Human Embryonic Palatal Mesenchyme (HEPM) cells in culture (Pratt et al., Teratogen. Carcinogen. Mutagen., 1982). The HEPM cell system has been reported to be complimentary to the tumor cell attachment assay.

Both assays will be run simultaneously at each contract laboratory. Forty to 50 chemicals, known teratogens and non-teratogens, will be selected and tested in each system for validation purposes.

MAJOR FINDINGS AND PROPOSED COURSE: The laboratory has improved the assay systems by standardizing assay conditions and identified certain objective criteria for judging an assay as valid. Testing has been completed on about 15 chemicals and testing on all 45 chemicals should be completed by September 1986.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Successful validation of the screening systems could help us to (1) accelerate the rate at which chemicals are evaluated for teratogenic potential; (2) improve the breadth of data on potential teratogens; (3) improve the selection of chemicals for whole animal testing; and (4) decrease testing cost.

BATTELLE PACIFIC NORTHWEST LABORATORIES
Seattle, Washington
(Interagency Agreement 1Y01-ES-4-0131-00)

TITLE: Inhalation Reproductive Toxicology Studies

CONTRACTOR'S PROJECT DIRECTOR: P.L. Hackett, Ph.D.

PROJECT OFFICERS (NIEHS): B.A. Schwetz, D.V.M., Ph.D.
(NIOSH): B.D. Hardin, Ph.D.

DATE CONTRACT INITIATED: September 14, 1984

CURRENT LEVEL: \$1,037,000

PROJECT DESCRIPTION

OBJECTIVE: This project is designed to provide the capacity to evaluate the potential of inhaled chemicals to cause developmental toxicity.

METHODS EMPLOYED: This contract provides capabilities for conducting studies to assess developmental toxicity through exposure of pregnant mice, rats, or rabbits to chemicals by the inhalation route. The manifestations of developmental toxicity which are included in this capability include classical structural teratogenicity as well as behavioral teratogenic effects and other adverse developmental or reproductive effects observed in the offspring of exposed females. The contractor's capabilities are diverse enough to permit observation of many different parameters of developmental toxicity, and the contract is designed to be flexible enough to permit the design of protocols which are tailored to the specific needs of chemicals to be tested.

MAJOR FINDINGS AND PROPOSED COURSE: This contract is designed to conduct the equivalent of classical teratology studies on three chemicals per year. The actual number of chemicals tested per year depends on the experimental design for any given chemical under test. The chemicals which have been identified as highest priority for testing under this contract include 1,3-butadiene, n-hexane, and tetrahydrofuran.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Large numbers of women of childbearing age are exposed to chemicals by the inhalation route both in the occupational setting as well as outside of the workplace. Chemicals which have been shown to be teratogenic in humans have also been shown to be teratogenic in laboratory animals. This contract provides the NTP with the capacity to evaluate the potential for chemicals to adversely affect the development of the unborn through the route of exposure which is the most relevant one for many chemicals, the inhalation route.

NORTHROP SERVICES, INC.
Research Triangle Park, NC 27709
(NIEHS N01-ES-4-5044)

TITLE: Animal Research on the Inhalation Toxicology of Environmental Chemicals

CONTRACTOR'S PROJECT DIRECTOR: Bernard Adkins, Jr., Ph.D.

PROJECT OFFICER (NIEHS): B.A. Schwetz, D.V.M., Ph.D.

DATE CONTRACT INITIATED: September 17, 1984

CURRENT LEVEL (5 YEARS): \$3,234,566

PROJECT DESCRIPTION

OBJECTIVE: Conduct research in the inhalation toxicology of environmental chemicals using dynamic flow-through inhalation chambers designed for use with small laboratory animals. Exposures are conducted intermittently because the inhalation facility is not equipped for 24-hour inhalation exposures. Generate, monitor, characterize, and control the generation of solid aerosols of asbestos and related natural and man-made fibers in 1-4 inhalation chambers as specified to support the research program of the National Toxicology Program and place into operation a computer-assisted augmentation of the existing gas inhalation facility based on concepts and specifications provided by the Government.

MAJOR FINDINGS AND PROPOSED COURSE: Documentation of the computer system has been started. One exposure room has been upgraded to handle more hazardous compounds. This room has been used to perform methyl isocyanate (MIC) exposures in support of numerous NTP investigators. Exposures of Strain A mice to ethylene oxide, vinyl chloride, and ethylene dibromide have resulted in concentration-dependent increases in the rate of formation of pulmonary adenomas. The 2-year tests of naphthalene and wollastonite have been completed. Plans are being made for continuing studies in the area of time-varying concentration as a determinant of inhalation toxicity and the initiation of studies of the inhalation teratology of toluene and chloroform.

SIGNIFICANCE OF BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The implementation of computer-assisted inhalation facility operation represents an attempt to bring inhalation technology into closer alignment with present-day laboratory computer technology. Computer-assisted operation greatly enhances the accuracy and flexibility of the operation of the inhalation facility as well as the documentation of that operation, and greatly reduces manpower requirements, thus reducing the labor costs of facility operation. The ability to conduct a technically difficult study such as MIC in a timely manner demonstrates the significance of this project to the program of the Institute.

NATIONAL ACADEMY OF SCIENCES
Washington, DC 20418
(NIEHS N01-ES-4-5058)

TITLE: Methods for the In-Vivo Toxicity Testing of Complex Mixtures from the Environment

CONTRACTOR'S PROJECT DIRECTOR: Devra Davis, Ph.D.

PROJECT OFFICER (NIEHS): B.A. Schwetz, D.V.M., Ph.D.

DATE CONTRACT INITIATED: September 26, 1984

CURRENT LEVEL: \$237,500

PROJECT DESCRIPTION

OBJECTIVE: This project is intended to support the preparation of a report which identifies scientific approaches for the systematic examination of the toxicity of complex mixtures.

METHODS EMPLOYED: The National Academy of Sciences selected a committee of experienced toxicologists who have been involved in the evaluation of the toxicity of complex mixtures. Based on their experiences and information from the literature, the committee will prepare a report which identifies an approach or approaches toward evaluating the toxicity of complex mixtures.

MAJOR FINDINGS AND PROPOSED COURSE: Individual members of the Academy Committee prepared reviews of previous evaluations of the toxicity of selected complex mixtures, including gasoline, cigarette smoke, diesel exhaust, and other complex mixtures of environmental concern. The goal of this review was to identify the approaches used and the strengths or limitations of the approaches for the individual mixtures that were tested. These data bases and approaches are now being evaluated closely to serve as examples for the preparation of guidelines for future evaluation of complex mixtures.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Procedures for evaluating the toxicity of complex mixtures have not been well characterized and the subject of testing mixtures has been highly controversial because of the difficulties of identifying scientifically acceptable approaches toward evaluating mixtures. In contrast to our reluctance to test mixtures, the real world of exposure to chemicals in the environment consists almost solely of exposure to mixtures rather than pure chemicals. Thus, an approach that is acceptable for the evaluation of the toxicity of mixtures would be a significant contribution to the armamentarium of toxicologists.

RESEARCH TRIANGLE INSTITUTE
Research Triangle Park, North Carolina 27709
(N01-ES-5-5080)

TITLE: Conventional Teratology Studies

CONTRACTOR'S PROJECT DIRECTOR: C. J. Price, Ph.D. and J. D. George, Ph.D.

PROJECT OFFICER (NIEHS): B.A. Schwetz, D.V.M., Ph.D.

DATE CONTRACT INITIATED: September 29, 1983

CURRENT LEVEL: \$597,000

PROJECT DESCRIPTION

OBJECTIVE: This project is designed to provide the capacity for evaluating the potential of chemicals to cause developmental toxicity (by routes of exposure other than inhalation).

METHODS EMPLOYED: Studies conducted under this protocol include those designed as structural teratology studies as well as studies designed to assess behavioral teratology or other functional alterations which result from in utero exposure to chemicals. Studies can be conducted in mice, rats, or rabbits. Any route of exposure can be used except for inhalation. This contract was started in the fourth quarter of 1983. Studies have been conducted or are in progress on theophylline, triethylene glycol dimethyl ether, nitrofurazone, diethylene glycol dimethyl ether, alpha-methyl dopa, codeine, scopolamine, ethylene glycol diethyl ether, and ethylene glycol. Additional chemicals which have been identified for evaluation under this contract include 1,1,1-trichloroethane, 2-ethyl hexanol, mono-ethylhexyl phthalate, hexachlorobutadiene, diethyl phthalate and dimethyl phthalate.

SIGNIFICANCE OF BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Developmental toxicity is one of the critical parameters in the objective of the National Toxicology Program to characterize the toxicity of chemicals. Women of childbearing age are routinely exposed to large numbers of chemicals during early pregnancy, many of which have not been adequately characterized for their potential to cause alterations in the development of the unborn. This contract is designed to provide data to predict the possibility that chemicals might cause developmental toxicity in humans.

PUBLICATIONS

Kimmel, C.A., Price, C.J., Sadler, B.M., Tyl, R.W. and Gerling, F.S. (1985) Comparison of distilled water (DW) and corn oil (C) vehicle controls from historical teratology study data. The Toxicologist 5(1), p. 185.

MEDICAL COLLEGE OF VIRGINIA/VIRGINIA COMMONWEALTH UNIVERSITY
Richmond, Virginia 23298
(N01-ES-5-5094)

TITLE: Examination of Immunotoxicity by Chemical Xenobiotics

CONTRACTOR'S PROJECT DIRECTOR: Albert E. Munson, Ph.D.

PROJECT OFFICER (NIEHS): M.I. Luster, Ph.D.
Immunotoxicology Group Leader, STR, TRTP

DATE CONTRACT INITIATED: May 1, 1985

CURRENT ANNUAL LEVEL: \$420,000

PROJECT DESCRIPTION

OBJECTIVE: The objective of this contract encompasses efforts to assess the presence and extent of immunotoxicity resulting from exposure to selected chemical xenobiotics. The potential of xenobiotics to influence immune function as well as to act as a sensitizer (haptens) will be determined. The ultimate goal of these studies is to determine whether such exposure represents a potential human health hazard. The major tasks involved include: (i) establishment and proficient demonstration of a standardized set of immunologic tests; (ii) examination of selected chemicals using these test systems; and (iii) examination of the cellular and molecular events associated with chemical-induced immunotoxicity.

METHODS EMPLOYED: Utilizing this screening panel (Table 1), 4 chemicals per year will be examined for immunomodulation and 4 chemicals per year as potential sensitizers. Chemical nominations will be provided by NIEHS, regulatory agencies or private organizations. Final chemicals selected will be determined by an ad hoc committee chaired by the Project Officer. Data obtained from this screen will allow for extrapolation with a reasonable degree of confidence regarding the safety of the drug or chemical for the immune system under the conditions defined. One chemical per year (total of 5) that are positive (immunotoxic) in the screen will be evaluated in detail as part of this contract using additional in vitro and in vivo assays to determine the cellular and molecular events associated with the onset of immunomodulation. Depending upon results obtained in the primary screen, this may involve combinations of any of the following: (a) hypersensitivity testing; (b) enumeration and function of lymphocyte subpopulations; (c) expanded host resistance studies; (d) tests for autoimmunity; (e) bone marrow function; (f) evaluation of structure-activity relationships; and (g) employment of various co-culture systems.

Table 1

Screening Panel for Detecting Immune Alteration Following
Xenobiotic Exposure in Rodents

Parameter	Procedures Performed
Immunopathology	Hematology - Complete blood count and differential Weights - body, spleen, thymus, kidney, liver Histology - spleen, thymus, bone marrow and target organs Spleen and bone marrow cellularity
Host resistance	Transplantable Tumor Cells <ul style="list-style-type: none"> • Growth of syngeneic PYB6 tumors • Development of B16F10 lung nodules Infectious Agents <ul style="list-style-type: none"> • Listeria challenge • EMC viral challenge • Plasmodium induced parasitemia
Lymphocyte enumeration	Enumeration of splenic B and T cells
Cell-mediated immunity	Lymphocyte blastogenesis to mitogens (PHA or Con A and LPS) and allogeneic leukocytes in mixed leukocyte culture (MLC) Natural killer cell cytotoxicity of tumor targets
Humoral immunity	Antibody plaque forming cell (PFC) response to sheep erythrocytes (direct and indirect)
Macrophage function	Quantitation of peritoneal macrophage cell number, phagocytic ability, and basal and activated ectoenzyme levels
Hypersensitivity skin testing of selected compounds (optional)	Ability to sensitize rodents using skin assays

MAJOR FINDINGS AND PROPOSED COURSE: Studies are presently underway to examine the influence of lithium carbonate on immune function. Glutaraldehyde is being examined as a potential sensitizer. No data is available as of yet.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A correlation has been clearly established between the administration of chemical immunosuppressants and an increased incidence of infectious diseases and neoplasia. The evidence for increased bacterial, viral, fungal and parasitic diseases in patients on chronic immunosuppressive chemicals has been well documented by Allen (Infection complicating neoplastic disease and cytotoxic therapy. In

Infection and the Compromised Host, 1976). Likewise, McKhann (Transplantation 8:209, 1971) observed that the incidence of cancer in renal transplant recipients on prolonged immunosuppressive chemotherapy was 4.6-6.1 times higher than in the general population.

Studies in laboratory animals also have supported these clinical observations and demonstrated an enhanced incidence of UV-induced or benzopyrene-induced cancer in mice treated with immunosuppressive agents. The mechanisms and relationship between altered host resistance and immune dysfunction is complex, poorly defined and of extreme importance. Chemicals of environmental concern have been recently shown to induce immunosuppression as evidenced by depressed antibody mediated immunity, cell-mediated immunity or M ϕ dysfunction in rodents following sublethal exposure. Some of the chemicals which induce immunologic effects in rodents include 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated biphenyls, polybrominated biphenyls, gallic acid, DES, BP, hexachlorobenzene, pentachlorophenol, certain organo and heavy metals. Some studies have indicated that exposure to certain chemicals can alter resistance to bacteria, viruses, parasites and transplantable tumor cells. Of major concern is the correlation of these immunologic findings with altered host susceptibility and the extrapolation of these chemically-induced immunobiologic effects to humans.

BIOMETRY AND RISK ASSESSMENT PROGRAM

BIOMETRY AND RISK ASSESSMENT PROGRAM Summary Statement

The Biometry and Risk Assessment Program (BRAP) plans and conducts basic and applied research in the areas of quantitative and biochemical risk assessment, statistics, biomathematics, and epidemiology. A major focus of this research effort is the qualitative and quantitative estimation of adverse health effects resulting from exposure to potentially hazardous environmental agents and the development of methodology useful in this estimation process. Attention is also directed toward the identification of environmental risk factors and the elucidation of the biological mechanisms that underlie their action. Due to the complexity of many of the issues under investigation, an increasing proportion of this research is being conducted on a program-wide basis, combining the scientific expertise found in its different organizational units.

In addition to conducting its own research effort, the BRAP also provides statistical, mathematical, data processing, and computer engineering support to other programs of the Institute. It assists the Office of the Director in addressing specific health issues that bear on the welfare of the general public; and maintains an active association with peer groups in other federal agencies, academic institutions and private organizations with similar research interests.

The Biometry and Risk Assessment Program is organized into an Applied Pathology Section within the Office of the Director, a Statistics and Biomathematics Branch (SBB), an Epidemiology Branch (EB), a Laboratory of Biochemical Risk Analysis (LBRA), and a Computer Technology Branch (CTB).

The Statistics and Biomathematics Branch conducts a broad research effort in a variety of areas such as the design and analysis of carcinogenicity experiments, statistical studies in genetic toxicology, mathematical modeling of various biological phenomena at the molecular level, and risk assessment methodology development. Branch scientists also provide a comprehensive consulting service for the research staff of the Institute.

The Epidemiology Branch initiates field studies of human disease, particularly chronic diseases, attributable to environmental pollutants; investigates the effects of environmental toxins on fetal and child development; and conducts basic and applied research in laboratory support methodology involved in the monitoring of human populations.

The Laboratory of Biochemical Risk Analysis is primarily concerned with the development of laboratory procedures for quantifying exposures in terms of the biologically effective dose, and with the application of these procedures to human population monitoring and to the enhanced extrapolation of toxicologic outcomes across species.

The Computer Technology Branch operates the Institutes's computer systems and the network of terminals connected to the various computers at NIH/DCRT; provides programming consultation services including software systems development to Institute personnel; maintains an active computer engineering group, which furnishes computing support to laboratory research activities in various branches; provides systems analysis and project management support to both Institute and NTP system development projects, and coordinates the Institutes's word processing and office automation activities.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 43009-02 BRAP

PERIOD COVERED

October 1, 1984, to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Role of Kidney & Nutritional Factors in Metabolism of Toxic & Essential Metals

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert A. Goyer Chief APS BRAP NIEHS

Others: Winona Victory Expert APS BRAP NIEHS
Chris R. Miller Bio. Lab. Techn. APS BRAP NIEHS
Shi-ya Zhu Visiting Fellow APS BRAP NIEHS

COOPERATING UNITS (if any)

LAB/BRANCH

Office of the Director, Biometry and Risk Assessment Program

SECTION

Applied Pathology Section (APS)

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

3

PROFESSIONAL

2

OTHER

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The cellular pathology of the kidney is studied in rats following exposure to toxic metals, lead or cadmium. In this phase of the study a series of experiments is being conducted to determine the influence of the toxic metals on essential trace metal metabolism. A number of metabolic parameters and indicators of toxicity are measured. These include cell ultrastructure, toxic and trace metal burden and excretion, changes in metal-binding protein, e.g., metallothionein, and functional changes in the renal tubule.

Results to date indicate that lead exposure increases urinary zinc in a dose and exposure length-dependent manner; urinary calcium is increased at the highest lead dose only. Cadmium exposure increases urinary zinc excretion with no effect on urinary calcium. Lead-exposure produces no change in tissue zinc content of most organs with the exception of testes and bone. Calcium content of kidney is normally low, but there is a sudden rise in calcium concentration if kidney lead concentration exceeds 60 $\mu\text{g/g}$. A preliminary estimate of a threshold for renal lead toxicity has been determined. Animals injected with cadmium have increased metallothionein content of liver and kidney with liver metallothionein concentration two-fold greater than kidney. The proportion of non-metallothionein bound cadmium in each organ is different with the kidney having the larger fraction.

The purpose of these studies is twofold: one is to determine parameters of renal toxicity in response to toxic metal exposure. The second is to determine the influence of dietary essential metal levels on toxicity in organs such as kidney, liver, and bone which preferentially accumulate toxic metals. From these studies the influence of various factors on risk to exposure to toxic metals may be estimated.

PROJECT DESCRIPTION

OBJECTIVES:

- (1) To determine the influence of toxic metals (lead, cadmium, etc.) on the metabolism and renal handling of essential trace metals (in particular, zinc, calcium and copper).
- (2) To understand how dietary constituents (in particular, essential trace elements) affect accumulation and toxicity of metals.
- (3) To determine sensitive measurements of renal function and structure which can detect toxic metal burdens prior to frank renal damage. These include measurements of tubular function by renal clearance and metabolic balance experiments.
- (4) The results are to be used to establish risk to exposure to toxic metals.

METHODS EMPLOYED: Young rats (Charles River CD) are exposed to toxic metal (lead or cadmium) at one of several doses for varying time periods. Essential minerals in the diet are varied by using one of several rat chows. Urinary metal excretion is determined from daily collections obtained with the animals in plastic metabolism cages. During these days, the animals may be treated in groups by: (1) altering dietary composition; (2) administering one of several chelating agents or drugs (such as diuretics) which affect toxic or essential metal excretion. Urinalysis is performed to measure: urine volume, excretion of protein, creatinine, several essential metals, the toxic metal, and other urinary constituents. Glomerular filtration rate, renal plasma flow and tubular transport functions are measured by standard clearance procedure. At termination of the exposure and test period, animals are killed to determine blood, plasma, and tissue content of essential and toxic metals. Analysis is by atomic absorption spectroscopy. Metallothionein is assayed by cadmium-hemoglobin saturation method and gel chromatography. Morphologic changes are assessed in tissues prepared for light and electron microscopy.

MAJOR FINDINGS AND PROPOSED COURSE:

- A. Effects of high level lead exposure on zinc and calcium excretion in rats: influence of chlorothiazide treatment. Rats consuming high mineral content chow and 10,000 ppm Pb for 12 weeks were studied for trace metal excretion while on Pb, subsequent to its removal from water, and during chlorothiazide treatment. Urine zinc was two- to three-fold higher than controls in Pb-exposed rats while on Pb and continued for several days after Pb removal. Chlorothiazide treatment in Pb-exposed rats produced a small increase in zinc excretion. Calcium excretion increased three- to four-fold when Pb was removed from drinking water. Chlorothiazide produced a greater decrease in calcium excretion and a larger increase in sodium excretion in lead-exposed rats. This enhanced sensitivity to diuretics resembles that observed in zinc-deficient rats.
- B. Dose-response relationships between levels of lead exposure and urinary and tissue metal content. Rats on a recommended mineral allowance diet were exposed to 0, 200, 500, or 1000 ppm Pb for periods up to 12 weeks. Urinary excretion of Pb and several essential metals was determined for a four-day

period after one, four, eight, or twelve weeks of exposure. Blood, kidney, brain, liver and urine showed dose-related increases in Pb concentration with the peak at four weeks. There was a dose-related increase in urine zinc but urine calcium was elevated at the highest dose only. No differences in sodium, potassium, or magnesium excretion were observed. Plasma and most tissue metal concentrations were not changed by lead exposure, except for a decrease in bone and testes zinc at high renal Pb concentrations, and an increase in kidney calcium. The mechanisms of these findings will be studied by evaluation of possible alterations in homeostatic regulation of zinc and calcium balance which affect renal and gastrointestinal transport pathways.

- C. Partitioning of renal zinc between metallothionein and EDTA during chelation therapy. There is a marked increase in renal zinc concentration in animals subsequent to chelation therapy with EDTA. Theorizing that this may reflect a redistribution of endogenous zinc within the kidney with respect to metallothionein, we studied the time course of changes in renal zinc and metallothionein subsequent to one- or three-day treatment with EDTA. We found that the increase in renal zinc can be accounted for by EDTA retention in the kidney for as long as one week. The cadmium-hemoglobin assay indicated some increase in metallothionein but gel chromatography revealed that metallothionein was decreased and there was a significant increase in small molecular weight zinc component which further study determined to be retained zinc-EDTA. We concluded that the Cd-hemoglobin method for analysis of metallothionein is subject to an upward distortion in the presence of EDTA. By depleting available zinc, EDTA greatly reduces the metallothionein in kidney within 24 hours. Although EDTA is rapidly excreted from the body, a small amount of EDTA is retained in the kidney. An evaluation of zinc distribution changes which may have occurred in liver will follow.
- D. Effects of length of cadmium exposure on liver and kidney cadmium distribution. Rats receiving daily cadmium injections were studied for mineral excretion rates and tissue distribution of metals and metallothionein after two, four, six or eight weeks exposure. Liver cadmium increased in a time-dependent manner until the eight-week sacrifice. Only a small fraction of liver cadmium was not bound to metallothionein (approximately ten to fifteen percent). Kidney cadmium showed a similar rate of increase but the fraction of the total cadmium which was not bound to metallothionein was much larger (on the order of 35 to 45 percent). The ratio of metallothionein-bound cadmium:non-metallothionein cadmium ranged between one and two in kidney while for liver the range was between four and eleven. The molar ratio of cadmium:zinc in kidney increased six-fold in kidney while liver increased only four-fold. Thus, there appears to be a greater change in the ratio of cadmium:zinc than the ratio of metallothionein-cadmium to non-metallothionein cadmium. Urinary zinc excretion increased in parallel with the increase in urine cadmium but there was no effect on urine calcium excretion. Experiments are being designed to determine more specifically the role of the cadmium:zinc ratio in the pathogenesis in cadmium nephropathy.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Dietary factors (especially calcium, magnesium and zinc) are now being linked to a number of human diseases, including renal disease, cardiovascular disease and diabetes mellitus. Our research seeks to understand the mechanisms by which toxic metal exposure (lead or cadmium) alters essential trace metal balance in animal models,

and how dietary minerals influence the toxic metal accumulation in the critical organ for toxicity, the kidney. Experimental data obtained will help to understand the following: (1) how lead movement in the 'calcium stream' may alter the many physiological processes normally controlled by calcium fluxes; (2) why toxic metals accumulate preferentially in the kidney; and (3) what sensitive indicators can be used to predict renal damage from toxic metal exposure. The results are to be used to establish risk to exposure to toxic metals.

PUBLICATIONS

Goyer, R.A.: Metal-Protein Complexes in Detoxification Processes. In: Clinical Chemistry and Clinical Toxicology of Metals. Proceedings of the 2nd International Conference on the Clinical Chemistry and Chemical Toxicology of Metals in Montreal, Canada, on July 19-22, 1983. Stanley S. Brown and John Savory (Eds.), Academic Press, Inc., London, 1983, p. 199-209.

Goyer, R.A.: Urinary System. In: Environmental Pathology. N.K. Mottet (Ed.), Oxford University Press, New York, 1985, p. 290-319.

Goyer, R.A., Cherian, M.G., and Delaquerriere-Richardson, L. Correlation of Parameters of Cadmium Exposure with Onset of Cadmium Induced Nephropathy in Rats. J. Environ. Pathol., Toxicol. and Oncology 5: 89-100, 1984.

Landrigan, P.J., Goyer, R.A., Clarkson, T.W., Sandler, D.P., Smith, J.H., Thun, M.J., and Wedeen, R.P.: The Work-Relatedness of Renal Disease. Arch. Environ. Health 39 (3): 225-230, May/June 1984.

Victory, W., Miller, C., Fowler, B.A.: Lead Accumulation by Rat Renal Brush Border Membrane Vesicles. J. Pharmacol. Exp. Ther. (3): 589-596, 1984.

COMPUTER TECHNOLOGY BRANCH

COMPUTER TECHNOLOGY BRANCH

Summary Statement

The Computer Technology Branch has the responsibility of providing computing, data processing, and office automation support to NIEHS and the National Toxicology Program. This service may be thought of as consisting of five cooperating and interdependent efforts, namely computer operations and support programming, information systems development, computer engineering, telecommunications support, and office automation development and management.

The computer operations and programming effort assumes the responsibility for maintaining NIEHS' two VAX 11/780 computers and a network of terminals connected to the various computers at NIH/DCRT, Parklawn, and NCTR, assisting the NIEHS community in its use of available computer systems, providing programming consultation services as required, providing software systems development capabilities to support intramural research efforts, and providing support and collaborative assistance to the computer engineering effort.

The information systems development effort consists of several projects for the development of large, automated systems for both the Institute and the NTP. Institute projects include efforts on behalf of the Office of Administrative Management, the Extramural Program, and the Office of Program Planning and Evaluation. For the NTP, ongoing projects include the Chemical Information and Tracking System, the Toxicology Data Management System (in cooperation with the National Center for Toxicological Research), and management of the Carcinogenesis Bioassay Data System.

Provision of computer engineering support to the laboratories of the Institute is also made available within the Computer Technology Branch. Solutions are being sought to engineering problems related to all aspects of computer hardware and software. Tasks within this effort have included the specification of personal computers, minicomputers and microcomputers, peripherals, and vendor-supplied software; the design of timing devices and interfaces between minicomputers and laboratory instruments; and the development of software for control of experiments, data acquisition, data analysis, and data transfer.

Telecommunications support consists of provision of hardware, software, and consulting services to the Institute in areas involving data transmission. Of recent interest have been extensive efforts designed to facilitate document exchange among the Institute's various word processors and computers and plans for expansion of access capabilities to DCRT. A major task continues to be providing assistance, documentation, and instructions to users who report configuration and/or operational problems (real or perceived) with telecommunications hardware, software, and access methods.

Office automation support to the Institute requires coordination with and complements the above detailed activities. Selection and acquisition of a comprehensive network of word processors has been accomplished, and ongoing related duties include management of the machines and provision of training for their users. Expansion of this support is continuing, with current efforts concentrating on acquisition of additional word processors and on selection and testing of centralized office automation functions which will reside on the VAX system.

DYNAMAC CORPORATION - ROCKVILLE, MD 20852
(N01-ES-2-8001)

TITLE: National Toxicology Program Computer Support

CONTRACTOR'S PROJECT DIRECTOR: Ms. Nancy Bonney

PROJECT OFFICER (NIEHS): Mr. R. M. Rowley, Computer Systems Analyst,
Computer Technology Branch, BRAP

DATE CONTRACT INITIATED: January 1, 1982

CURRENT ANNUAL LEVEL: \$1,207,439

PROJECT DESCRIPTION

OBJECTIVES: The major objective of this contract is to provide the data entry, computer programming, and coding support required to produce technical reports for the NTP Carcinogenesis Testing Program.

METHODS EMPLOYED: The standard nomenclature of the Pathology Code Dictionary is used to code tumor diagnoses for rats, mice and hamsters in all completed bioassays. These codes are then keyed onto computer files on the DCRT computer. Computer programs are then run against the data to produce pathology tables and statistical reports which are used in the NTP Technical Reports.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE NTP: The Carcinogenesis Testing Program is the major testing program of the NTP and is responsible for determining the carcinogenicity/toxicity of hundreds of chemical compounds to which the population of the U. S. is exposed annually. The Technical Reports on these chemicals are the official NTP publications describing the results of this testing. The pathology and statistical tables produced by the contractor are included in the reports and are the primary data source used in evaluating the carcinogenicity/toxicity of the chemical.

EPIDEMIOLOGY BRANCH

EPIDEMIOLOGY BRANCH Summary Statement

The potential effects of chronic exposure to low-dose environmental chemicals on human health are more diverse than any single epidemiologic strategy can encompass. The Epidemiology Branch has emphasized innovative methods and interdisciplinary collaborations in developing its program of research. This effort draws on the expertise of laboratory scientists, clinical scientists, and statisticians available within our own Program. This combination of scientific disciplines provides a valuable resource for carrying out epidemiologic research. The breadth of this research effort is indicated in the examples below.

New laboratory methods can expand the power of epidemiologic studies to define exposures and disease outcomes. The development and application of these laboratory methods are greatly aided by our collaboration with the Laboratory of Biochemical Risk Analysis (LBRA), and also with laboratories outside NIEHS. For example, a new post-labeling assay for the detection of DNA adducts has been used to look for adducts in human placentas. This has led to the first clear demonstration of an association between cigarette smoking and DNA adducts in humans. This work may ultimately lead to the identification of specific chemicals in cigarette smoke (as well as in other environmental exposures) that damage human genetic material.

Scientists in the Epidemiology Branch select specific diseases for study that are suspected to be related to environmental toxins, but which have not been carefully studied before. For example, chronic renal failure is a serious, debilitating and expensive disease that for the most part occurs without a known cause. A few specific environmental exposures like lead are known to be toxic to the kidneys at high doses, but the contribution of low-dose environmental factors over many years is not known. A case-control study of 709 patients with chronic renal disease is being carried out to investigate a broad range of environmental exposures for possible renal toxicity.

Reproduction has been singled out as a biological process that may be particularly susceptible to injury by environmental exposures. Animal studies suggest that early pregnancy may be a time of particular vulnerability to toxins. Recent developments in laboratory assays for early pregnancy make it practical for the first time to conduct epidemiologic studies of early pregnancy loss. In a prospective study of 230 women who are trying to become pregnant, daily urine specimens are being tested for pregnancy hormone in order to detect subclinical pregnancy loss. The risk of such loss will be studied in relation to alcohol consumption, smoking, medications and other common exposures.

Animal experiments are frequently used to suggest biological pathways of effects that may occur in humans. For example, fertility in animals is disturbed by exposure to many chemicals, but the susceptibility of human fertility to impairment has not been well studied. This has prompted new methods for measuring the effect of environmental exposures on fertility in humans. In one feasibility study, the time required for women to become pregnant was used to estimate the monthly probability of conception for 678 women. Women who smoked were found to have significantly increased time to conception. This method is being further developed for the study of occupational and other environmental exposures.

Finally, environmental accidents can sometimes provide rare opportunities to examine the effect of particular chemical exposures on persons who are accidentally poisoned. Polychlorinated biphenyls (PCBs) are toxic and ubiquitous hydrocarbons but seldom are found in high doses. An accidental poisoning of 2000 persons in Taiwan presented the opportunity for the study of children born to poisoned mothers. 108 exposed children and 106 controls have been given neurologic, genetic, dental and dermatologic exams. This study should enable a detailed description of the congenital and childhood abnormalities associated with high prenatal exposures to PCBs.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 43002-09 EB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Breast Milk and Formula Project: Exposure to Halogenated Aromatic Compounds		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> Walter J. Rogan Medical Officer EB NIEHS </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> Beth C. Gladen Statistician SBB NIEHS </div> <div style="display: flex; justify-content: space-between;"> Gwen T. Waldman Staff Fellow EB NIEHS </div>		
COOPERATING UNITS (if any) Laboratory of Biomedical Risk Analysis, Biomathematics Branch, System Toxicology Branch, Wake Area Health Education Center, Raleigh, NC; Durham Women's Clinic, Durham, NC; East Carolina School of Medicine, Greenville, NC; National Taiwan University Hospital, Taipei, Taiwan, ROC		
LAB/BRANCH Epidemiology Branch		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS <div style="text-align: center;">2.0</div>	PROFESSIONAL <div style="text-align: center;">2.0</div>	OTHER <div style="text-align: center;">.0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (a1) Minors <input checked="" type="checkbox"/> (a2) Interviews </div> <div> <input checked="" type="checkbox"/> (b) Human tissues </div> <div> <input type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Polychlorinated biphenyls (PCBs) and DDT are toxic, widespread hydrocarbons that are poorly understood in terms of their toxicity for human beings. Both of these chemicals pass from mother to child across the placenta and through breast milk. This project includes three studies of subjects exposed to either low levels of these compounds in the US or to higher doses in Taiwan. Two studies are of children and one is on adults.</p> <p>The Breast Milk and Formula study is a birth cohort follow up study of 856 North Carolina children. PCBs and DDE (the stored metabolite of DDT) are measured in breast milk and the children are followed medically over time. Most of the children have completed 5 years of observation and are now followed only by a birthday card registry. An early finding from the study is that women with higher levels of DDE breast feed for shorter lengths of time. Levels of DDE are higher in countries with active spraying programs.</p> <p>An epidemic of 2000 cases of PCB poisoning occurred in Taiwan in 1979. Rice oil was accidentally contaminated during manufacture. We did a survey of 108 children who were born to mothers who were poisoned, 40 of their older siblings, and 106 controls. All children received a physical examination and the mothers answered a questionnaire about their children's health.</p> <p>Another study of this outbreak will use a modification of an assay for sister chromatid exchanges to evaluate subtle genetic damage and also investigate the immunologic changes associated with exposure to the contaminated oil (see also Z01 ES 46003-01 LBRA).</p>		

PROJECT DESCRIPTION

METHODS EMPLOYED: 1) The Breast Milk and Formula study began as a clinical follow up study of children exposed through breast milk to PCBs and DDT. The children had medical histories and physical examinations done serially from birth through early childhood, and the amount of PCB and DDT in breast milk was measured. Clinical examinations stop at age five; from then on, the children are followed by mail once a year to keep addresses up to date. About 700 of the 900 original children are now five years old or older. In the data from this study, women with higher levels of DDT breast feed for shorter lengths of time. 2) The Children's survey done in Taiwan included neurologic, genetic, dental, and dermatologic examinations. Blood and cerumen was collected for PCB analysis and for studies of immune function on selected children; spot urines were collected for possible uroporphyrin analysis. 3) The study of adult women in Taiwan uses two kinds of laboratory assays: a modification of an assay for sister chromatid exchanges that measures subtle genetic damage, and immune function studies that measure T and B cell populations and other markers of immune status.

MAJOR FINDINGS AND PROPOSED COURSE: 1) In the Breast Milk and Formula study data, we have found that PCB and DDE levels decline over time spent lactating, and that women with higher levels of DDE breast feed for shorter lengths of time. Maternal blood samples have higher levels than cord blood or placenta. The major remaining objectives are cohort preservation and data analysis. We have specific hypotheses about morbidity, growth and development. Hypotheses about long term effects and the description and validation of alternate chemical methods are lower priority. Of the original study hypotheses, only behavior in school and school achievement lack some data, but this should be obtainable simply from the registry. In order to extend and replicate the association of shorter duration of breast feeding and higher DDT levels, we hope to conduct a short term follow up study of duration of breast feeding in an area where DDT levels are much higher than in current study. 2) The Taiwan studies are just finished with data collection, and no findings are yet available. For the children's survey, we plan to have the blood samples collected in the field analyzed for PCBs and their contaminant chemicals. 3) Laboratory analyses of sister chromatid exchanges and immune function are underway in the adult study.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAMS OF THE INSTITUTE: PCBs and DDT have now become worldwide pollutants, and all human beings are exposed to small amounts of them. These studies attempt to define how toxicity is expressed at relatively high doses, and also to see whether subtler forms of toxicity are present in the general population without special exposures. Although neither PCBs nor DDT are used in the US now, the chemicals are stable and substantial amounts remain in the environment. Continued human exposure is certain. In a broader sense, understanding the mechanism by which these chemicals produce illness may lead to better methods of prevention of drug or chemical induced illness.

PUBLICATIONS

Rogan, W.J., Gladen, B.C.: The study of human lactation for effects of environmental contaminants. Environ Hlth Perspec, 60:215-21, 1985.

Rogan, W.J.: Intrauterine and breast milk exposures. In Finberg, L. (ed.) Chemical Pollution and Children. CRC, in press.

Rogan, W.J., Gladen, B.C., Wilcox, A.J.: Potential reproductive and postnatal morbidity from exposure to polychlorinated biphenyls. Environ Hlth Perspec, 60:233-40, 1985.

Rogan, W.J., McKinney, B.C., Carreras, J.D., Hardy, N., Thullen, J.D., Tingelstad, J., Tully, M.: Polychlorinated biphenyls (PCBs) and Dichlorodiphenyl Dichloroethene (DDE) in human milk: effects of maternal factors and previous lactation. Amer J Pub Hlth, in press.

Rogan, W.J.: Epidemiology of environmental contaminants in breast milk in Proceedings of the conference on Maternal Environmental Factors in Human Lactation. Plenum, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 43004-07 EB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Environmental Exposures and Chronic Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Dale P. Sandler Senior Staff Fellow EB NIEHS

Walter J. Rogan Medical Officer EB NIEHS

Gwen T. Waldman Staff Fellow EB NIEHS

COOPERATING UNITS (if any) Bowman Gray School of Medicine/Baptist Hospital, Duke University Medical Center, University of North Carolina Medical School, Charlotte Memorial Hospital, Food and Drug Administration, Centers for Disease Control

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

1.75

PROFESSIONAL

.75

OTHER

1.00

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Chronic diseases contribute substantially to the morbidity of human populations and result in significant expenditures of public health dollars. Environmental agents may produce some of this disease, and identification of associations between exposures and certain diseases would presumably lead to the prevention of morbidity. Other than cancer, few chronic diseases have received much attention in studies of environmental hazards. The program in environmental exposures and chronic disease addresses the role of environmental factors in the etiology of some less well studied chronic diseases. The program is developing methodologies appropriate to the epidemiologic study of chronic diseases which are often difficult to characterize or define precisely, and is adapting methodologies that have been used in studies of cancer for use in studies of nonmalignant diseases. Current emphasis is on identifying risk factors for chronic renal disease. There are more than 50,000 individuals in the U.S. on maintenance dialysis for end stage renal disease at a cost of more than \$1.5 billion per year. Dialysis patients represent only a fraction of those with some form of renal dysfunction. A multi-center case-control study of risk factors for chronic renal failure is nearing completion, as is a case-control study of risk factors for biopsy diagnosed IgA nephropathy. Related studies involve the development of a renal disease classification scheme for use in etiologic studies, and the analysis of vital statistics and NIOSH Occupational Hazards Survey data to identify time trends, geographic patterns of renal disease and occupations with potentially increased renal disease risk. Projects in other areas include analysis of data from the Health and Nutrition Examination Survey to develop a new estimate of the number of persons who have been exposed to asbestos.

PROJECT DESCRIPTION

METHODS EMPLOYED: (1) A study of risk factors for chronic renal failure is nearing completion. The study uses a case-control epidemiologic design to identify risk factors and to clarify the relative importance of different exposures in determining disease risk. Cases are patients discharged from one of four participating hospitals with a new diagnosis of chronic renal disease. Disease is defined on the basis of renal dysfunction (serum creatinine > 1.5), and cases are limited to those in whom dysfunction can be determined to be of intrarenal origin. Controls are healthy persons selected from the community by random telephone screening or from Social Security/Medicare listings, who are similar to cases in age, race, sex and area of residence. Cases and controls are interviewed by telephone. Some of the environmental and occupational exposures of interest are lead, cadmium, solvents, analgesic drugs, non-steroidal anti-inflammatory drugs and antibiotics. (2) A study of risk factors for IgA nephropathy is also nearing completion. Cases are identified from pathology records at UNC Memorial Hospital, and include all individuals with a pathologic diagnosis of IgA nephropathy. These include a wide range of clinical diagnoses from benign hematuria to end stage disease. Controls are identified by random telephone screening. Cases and controls are interviewed by telephone concerning prior exposures and diseases. The questionnaire emphasizes occupational and environmental exposures such as solvents and metals, and history of infectious disease and allergy. Also, hospital records are being used to develop a renal disease classification scheme that can be used in epidemiologic studies. This will clarify methodologic issues in the design of studies of chronic renal disease. An algorithm for classifying patients on the basis of clinical and laboratory data is being developed. (3) Analysis of vital statistics, demographic and other population data often leads to important clues linking environmental exposures to specific diseases. To this end, several projects exploring various chronic diseases are underway. (a) A multivariate analysis of census data, vital statistics data, and data from a special occupational census is being carried out in an attempt to identify reasons for an apparent excess of mortality from chronic renal disease in the southern U.S. (b) Data from a study completed elsewhere are being used to estimate the prevalence of analgesic use and abuse in a Southern community, and to identify correlates of analgesic abuse. Analgesic abuse is purportedly more common in the Southeast than elsewhere in the U.S. Because analgesics may cause renal dysfunction, greater use of analgesics may help explain the higher mortality from certain forms of renal disease in the Southeast. (c) The x-rays of all persons in the Health and Nutrition Examination Survey that were read as positive for pleural disease and two negative films for each positive one have each been re-read by three specialized readers who were blinded to the HANES diagnosis. Case and control films were from individuals who were matched according to age, race, sex and region of residence. Results of this independent reading of the chest x-rays are being compared with the initial HANES diagnosis to determine the usefulness of the HANES data for estimating the prevalence of asbestos related pleural disease in the U.S.

MAJOR FINDINGS AND PROPOSED COURSE: (1) A total of 4289 patients had a discharge diagnosis suggesting chronic renal disease. Hospital charts were

reviewed for approximately 1000 of these who met simple criteria for study eligibility. Chart abstracts and full medical records were reviewed by collaborating nephrologists and 709 patients were ultimately included in the study. 709 controls were selected from social security listings and random telephone screening. More than 70% of the cases and controls have been interviewed. Many of the remaining subjects were found to be deceased and next-of-kin are being traced and will be asked to provide exposure information. Data analysis will begin in the fall of 1985. (2) Of 70 cases with biopsy diagnosed IgA nephropathy that were identified, 52 subjects were found eligible for study. Controls were selected by telephone screening. Interviews have been completed with 90% of the cases and controls. Data are being coded and edited and analysis will begin shortly. (3) Data from hospital records of 709 patients with chronic renal disease are being analyzed. Data suggest that diagnostic criteria are highly variable and that patient work-ups are dependent upon a number of factors besides level of renal dysfunction. Only approximately 7% of patients had a biopsy but biopsy rates varied from 4% to 12% at the four hospitals. Other aspects of the work-up also varied by hospital which influenced the clinical diagnoses obtained. These data suggest the need for standardized work-ups, because the different approaches at different hospitals makes the classification of renal patients difficult. An attempt will be made to use these data to develop an algorithm for classification, but other approaches will also be evaluated. (4) Over 4500 patients with elevated serum creatinine values were identified through review of clinical laboratory data at one hospital. A random sample of these were selected to determine the proportion who were previously identified through review of discharge diagnoses and the proportion of new renal disease patients who would be missed using other case ascertainment approaches. Twenty percent of patients with abnormal laboratory values also had a renal disease discharge diagnosis and 20% of these had qualified for the study. Of the remaining patients with no renal discharge diagnosis, only 3% would have qualified. The extra effort entailed in reviewing charts of all patients with elevated creatinine values may be prohibitive in future studies, but a significant proportion of patients with dysfunction will be missed if this is not done. (5)(a) Analysis of a preliminary data set suggests that chronic renal failure mortality is elevated in the southeastern U.S. Within the limitations of this type of data, this excess appears unrelated to infection or hypertension prevalence. Mortality is greatest in counties where residents are poorly educated or of moderate income, and in counties of moderate size. Several occupations appear to be associated with increased mortality from renal disease. Analyses are being repeated on a larger data set to rule out potential confounding by other factors or chance. (b) Data from a study of hypertension and drug use in a total community are being analyzed. Preliminary analysis suggests that regular analgesic consumption is widespread in the South but varies greatly with race and sex. Future analysis will focus on additional correlates of analgesic consumption. (c) Approximately 1000 chest x-rays have been re-read by NIOSH class B readers. Preliminary data analysis suggests that the original Hanes diagnosis of pleural disease may not be accurate. Estimates of the prevalence of asbestos related disease will be made based on a refined classification of the films.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Standard epidemiologic methods have been useful in understanding etiologic agents in chronic diseases. The standard methods of epidemiology have, however, not been used extensively in studies of environmental exposures and human disease other than cancer. These studies all examine fairly widespread exposures that have known or suspected association with disease in humans. Documentation of these kinds of associations allows preventive intervention or risk modifications by decreasing exposure. Since the exposures studied here are common, the potential for significant disease reduction seems high. Methods of case ascertainment and classification developed for the renal disease studies should facilitate additional studies of risk for renal disease or other chronic diseases that are equally difficult to define. The completed studies should focus attention on environmental and preventive aspects of renal disease and other chronic diseases.

PUBLICATIONS

Rogan, W.J., Yang, G., Kimbrough, R.D.: Aflatoxin and Reye Syndrome: a study of livers from deceased cases. Archives of Environmental Health, 40:91-95, 1985.

Rogan, W.J.: Relationship of Surrogate measures to actual dose. In L. Gordis, (Ed.): Risk Assessment and Public Policy. Oxford Univ. Press, in press.

Sandler, D.P.: Methodologic issues in epidemiologic studies of renal disease: Criteria for case ascertainment and classification. Royal Society of Medicine, International Congress and Symposium series, in press.

Goyer, R.A. and Rogan, W.J.: When does a biologic change indicate disease? in Health Effects of Hazards Chemicals, Princeton, Princeton Scientific Publishers, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 43008-06 EB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Biochemical and Cellular Environmental Epidemiology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Richard B. Everson

Medical Officer

EB

NIEHS

COOPERATING UNITS (if any) Columbia University, U.S. Department of Agriculture Western Regional Research Center, University of North Carolina at Chapel Hill, Duke University, Baylor College of Medicine, Laboratory of Biochemical Risk Analysis, Laboratory of Pharmacology, NIEHS

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

1.0

PROFESSIONAL

0.8

OTHER

0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type Do not exceed the space provided)

This project seeks evidence of adverse effects associated with environmental exposures using a clinical research approach integrating laboratory and epidemiologic methods. It emphasizes interdisciplinary development of ideas and methodologies coupled with attention to details of both the laboratory procedures and the gathering and analysis of data concerning human subjects. Current effort focuses on the development of techniques for identifying genetic damage and alterations in metabolism associated with human exposure to potentially toxic substances. These techniques are being used in model studies of individuals exposed to known amounts of carcinogenic and mutagenic agents used for cancer chemotherapy, smokers, and individuals accidentally exposed to large quantities of PCBs. These studies are designed to help evaluate and refine assay methods, to investigate mechanisms involved in specific models of human disease, and to investigate the effects of exposures that may be important to public health.

PROJECT DESCRIPTION

METHODS EMPLOYED: Laboratory approaches to the detection of effects of toxic substances on human genetic material and metabolic function are being studied in field studies of model exposed populations. Patients exposed to known quantities of mutagenic drugs used for cancer chemotherapy are being used to study the capability of existing and newly developed laboratory techniques for measuring the extent and persistence of somatic, germinal, or heritable genetic damage. Laboratory studies included assays for resistance to 6-thioguanine of human lymphocytes; cytogenetic studies including the presence of micronuclei in the peripheral blood of splenectomized patients and, in non-splenectomized subjects, traditional assays for aberrations and sister chromatid exchanges; and assays of DNA for strand breaks and chemical adducts. The effects of medical, occupational, lifestyle, and environmental exposures on in vivo human metabolism are being investigated by assays of mixed function oxidase enzymatic activity in human placental tissue and mononuclear blood cells. In the metabolism studies populations include both a cross section of active, passive, and nonsmoking exposed women delivering at a university hospital in North Carolina and women accidentally exposed to rice oil contaminated with large quantities of PCB's in Taiwan. Assays include fluorometric determination of benzo(a)pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activity, HPLC profiles of benzo(a)pyrene metabolism, and Western blots for immunological detection of cytochrome P450's. The North Carolina cohorts of women are also being studied for the presence of DNA adducts in placental tissue and sister chromatid exchanges in lymphocyte specimens to study both these endpoints themselves and interrelationships of these endpoints with findings in the metabolism assays.

MAJOR FINDINGS AND PROPOSED COURSE: Quantitation of micronuclei in peripheral blood of splenectomized patients undergoing cytotoxic therapy was found to be a cost effective and very sensitive indicator of cytogenetic damage. Further field studies clarifying effects of residual spleen tissue on results for this assay are underway and studies seeking effects of lifestyle, dietary, and other environmental exposures are being planned. The assay for DNA strand breaks was found to be relatively insensitive to in vivo effects of cytotoxic drugs, although it is rapid and has good potential for automation. Further studies should probably focus on measuring in vitro sensitivity with this technique. For the metabolism studies over 200 specimens of human placenta have been analyzed for benzo(a)pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activity. Metabolic activity was strongly induced in specimens from subjects accidentally exposed to polychlorinated biphenyls in contaminated rice oil. This induction was present even though specimens were obtained some four years after exposure, demonstrating the ability of this class of widespread environmental contaminants to cause substantial and persistent effects on human metabolism. Further characterization of the specific biochemical alterations and long term monitoring of health effects in this population should be undertaken. Data from the studies of DNA adducts provided the first clear demonstration of an association between cigarette smoking and the in vivo formation of covalent DNA adducts in man. The study was the first report of the application of a novel postlabeling assay for detecting in vivo formation of DNA adducts in human tissue, and compared this with newly developed immunologic

assays. It suggests that the major adducts being formed by cigarette smoking in vivo may not be caused by several of the most intensely studied polycyclic aromatic hydrocarbons or amines in cigarette smoke. This work may ultimately lead to the definition of chemical components of cigarette smoke (as well as other environmental exposures) that most severely damage human DNA.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In a susceptible host, environmental exposures act through some biologic mechanism to produce disease. In the past, the laboratory has been of great help in defining each of these factors (susceptibility, exposure, mechanism, and outcome) in studies of infectious disease. In recent years, development of laboratory systems for measuring certain aspects of each of these factors, as they relate to the chronic diseases, has been rapid and exciting, especially in the area of genetic toxicology. Currently or in the near future, it may be anticipated that capabilities will exist to measure exposures to xenobiotics in the ppb range or lower, to classify genetic susceptibility by DNA repair capabilities, to seek biochemical mechanisms for events now related only phenomenologically, and to assess risk by observing direct effects on DNA or somatic cell mutation.

Applications of these tests to human populations, however, will be a difficult and complex undertaking. Test validation will be necessary, both in terms of its biologic meaning and of the more traditional biostatistical concepts of sensitivity and specificity. Details of both the laboratory procedures employed and subjects tested will require equivalent attention, preferably by scientists or groups of scientists with inter-disciplinary backgrounds and an understanding of both the test and the populations tested. Many factors concerning the subjects tested will require consideration, including evaluation of susceptibility and past exposures other than the specific exposure under study. A program aimed at developing both laboratory methods and epidemiologic methods that use the laboratory effectively should be of great utility in this undertaking.

PUBLICATIONS

Huel, G., Everson, R.B., Menger, I.: Increased hair cadmium in newborns of women occupationally exposed to heavy metals. *Environ Res* 35:115-121, 1984.

Everson, R.B., Flack, P.M., Milne, K.L., Warburton, D., Buchanan, P.D.: Mutagenesis assays of human amniotic fluids. *Environmental Mutagenesis* 7:171-184, 1985.

Everson, R.B., Ratcliffe, J.M., Flack, P.M., Watanabe, A.S., Hoffman D.M. Detection of low levels of urinary mutagen excretion by chemotherapy workers which was not related to occupational drug exposures. *Cancer Res* (in press).

Everson, R.B.: Detection of occupational and environmental exposures by bacterial mutagenesis assays of human body fluids. *J Occup Med* (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 44003-08 EB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Epidemiologic Study of Reproductive Outcomes and Environmental Exposures		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
Allen J. Wilcox	Acting Chief	EB NIEHS
Donna D. Baird	Senior Staff Fellow	EB NIEHS
Beth C. Gladen	Statistician	SBB NIEHS
Clarice R. Weinberg	Statistician	SBB NIEHS
COOPERATING UNITS (if any) Developmental Endocrinology Branch and Biometry Branch, National Institute of Child Health and Human Development, Columbia University, Atlanta University		
LAB/BRANCH Epidemiology Branch		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, NC 27709		
TOTAL MAN-YEARS 3.65	PROFESSIONAL: 2.65	OTHER: 1.00
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input checked="" type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The reproductive epidemiology project emphasizes the development and application of new methods for measuring human reproductive damage. Reproductive outcomes include fertility, sub-clinical early fetal loss, spontaneous abortion, fetal growth, and birthweight. Each of these outcomes can be affected by environmental factors, and represents a possible endpoint for studying the effects of toxins on human reproduction. One major component of this project is the study of fertility. Time-to-pregnancy is being developed as a potentially useful and sensitive measure of fertility. For example, a strong relation between smoking and time to pregnancy has been documented. Another approach to the measurement of fertility involves the study of daily urine specimens from women who are trying to become pregnant. By comparing the ovarian hormones in cycles which results in pregnancy with hormones in infertile cycles, new methods may be developed for measuring fertility impairment. These methods may be applicable to women not actively trying to conceive. A second component of this project is the study of very early pregnancy loss. In a prospective study of 230 women who have stopped using birth control in order to become pregnant, daily urine specimens are being collected to be tested for evidence of pregnancy. This will provide the best available estimate of the extent of early pregnancy loss in humans. The risk of early loss will be studied in relation to common exposures in this population, such as use of alcohol, tobacco, caffeine beverages and medications. Work continues on the development of a new method for the analysis of birth weight and perinatal mortality.		

PROJECT DESCRIPTION

METHODS EMPLOYED: The purpose of this project is to develop innovative epidemiologic methods for measuring human reproductive outcomes, and then to apply these methods to the study of environmental hazards. In particular, effort has focused on measurements of human fertility, measurement of sub-clinical early fetal loss, and development of a new method for analyzing birthweight.

(1) Measurement of human fertility. It is plausible that infertility can be caused by environmental chemicals. Actual environmental effects on fertility have been hard to measure, in part because the existing epidemiologic methods are insensitive. We are developing several approaches to measuring fertility. In one, we calculate the monthly probability of conception based on the length of time a group of women requires to become pregnant. In another, we are analyzing daily urine specimens from reproductive-age women. These urines will be analyzed for metabolic products of estrogen and progesterone, which reflect the function of the ovary and the corpus luteum. Urine specimens are available for both pregnant and non-pregnant cycles. This will allow us to characterize physiologic differences between fertile and non-fertile cycles in a way that has not been possible before. This may lead to improved methods for detecting environmental disruptions of human fertility in exposed populations.

(2) Measurement of early pregnancy loss. The risk of spontaneous abortion is known to be affected by environmental exposures. It is not known how much pregnancy loss occurs before pregnancy is recognized, or how sensitive this early loss might be to environmental exposures. In order to explore these questions, we are conducting a prospective study of 230 normal women who are trying to become pregnant. These women collect daily urine specimens that are analyzed for human chorionic gonadotropin, a pregnancy hormone produced about 7-8 days after conception. The risk of early loss in this group of women will be analyzed in relation to alcohol consumption, cigarette smoking, and other exposures common among these volunteers.

(3) Measurement and analysis of birthweight. Birthweight is an endpoint of interest for environmental studies because it is easily measured and strongly related to perinatal mortality. We are continuing to develop our model for analyzing birthweight. Our model considers the birthweight distribution and weight-specific mortality each as continuous variables with characteristic parameters. We show that weight-specific mortality is important not in its relation to absolute birthweight but rather to the birthweight distribution from which it is derived. This approach leads to fundamental reassessment of our understanding of birthweight. For example, we find that the overall smaller size of black infants is not a cause of higher black mortality, and may reflect genetic racial differences unrelated to fetal health. We are collaborating with NICHD in the procurement of detailed birthweight records from selected US states and foreign countries, which will provide us with extensive data on which to apply our methods.

MAJOR FINDINGS AND PROPOSED COURSE: (1) We tested the feasibility of measuring time to pregnancy as a measure of fertility in a study of 678 women. We found that among these women, cigarette smoking had a strong effect on fertility: women who smoked were only 72% as likely as non-smokers to get pregnant in any given cycle. Furthermore, this study indicates that time to pregnancy may be a more sensitive measure of fertility than other measures employed in the past. We calculate that with this method we would have detected the effect of smoking with a study of only 150 women. We are proceeding to develop this method in two ways: we are planning additional studies in more diverse populations to test how sensitive this measure might be to other environmental exposures, and we are developing improved statistical methods for analyzing these data. This approach may be useful not only for the study of fertility in itself, but also as a general screening mechanism for detecting reproductive hazards in the workplace or elsewhere. (2) Our prospective study of early pregnancy loss will be completed in the next year. This data set will be used to estimate the occurrence of early pregnancy loss, and to investigate how sensitive such loss might be to environmental exposures. This is the first study of its type, and thus offers a prototype for measuring early loss in other populations. (3) We have shown how the effects of racially-determined genetic differences in birthweight can be taken into account when analyzing birthweight and perinatal mortality. This is the first step towards isolating the possible effects of environmental hazards on fetal growth and survival.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Human reproductive outcomes are highly plausible endpoints for the study of environmental exposures. However, these outcomes are persistently difficult to measure, analyze, and interpret. This project intends to develop and strengthen the tools for measuring and analyzing fertility, sub-clinical fetal loss, spontaneous abortion and birthweight. As methods are developed, they are applied in studies of specific environmental exposures. In so doing, damaging effects of environmental factors on human reproduction can be recognized and averted.

PUBLICATIONS

Wilcox, A.J. and Horney, L.F.: Accuracy of spontaneous abortion recall. Am J Epidemiol, 1984; 120:727-733.

Baird, D.D. and Wilcox, A.J.: Cigarette smoking associated with delayed conception. JAMA, 1985; 253:2979-2983.

Wilcox, A.J.: Quantitative effects of chemicals on fertility. In Methods for Estimating Risk in Man and Chemical Injury in Non-Human Biota and Ecosystems, VB Vouk, ed., John Wiley and Son, New York, 1985; 457-476.

Wilcox, A.J., Weinberg, C.R., Wehmann, R.E., Armstrong, E.G., Canfield, R.E., Nisula, B.C.: Measuring early pregnancy loss: laboratory and field methods. Fertil and Steril, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 46002-01 EB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Environmental Exposures and Cancer Risk

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator) (Name, title, laboratory, and institute affiliation)

Dale P. Sandler Senior Staff Fellow EB NIEHS

Richard B. Everson Medical Officer EB NIEHS

Allen J. Wilcox Acting Chief EB NIEHS

Gwen T. Waldman Staff Fellow EB NIEHS

COOPERATING UNITS (if any) University of Minnesota, Harvard University, Cancer and Leukemia Group B member institutions, The Johns Hopkins University Training Center for Public Health Research, Laboratory of Biochemical Risk Analysis, NIEHS

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS

2.3

PROFESSIONAL

1.55

OTHER

.75

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The study of cancer risk is a major component of environmental epidemiology. The Epidemiology Branch emphasizes the development of methods for assessing environmental exposures, and uses biochemical measures of exposure or disease markers to evaluate cancer risk. A study of the relationship of childhood and/or adulthood passive exposure to cigarette smoke and cancer risk has been completed, and data collected for this study are now being used to evaluate the quality of exposure histories obtained from surrogate respondents. Additional studies of "passive smoking" have been started. These include a prospective study of mortality and cancer incidence among persons who were living with smokers in 1963, and measurement of indicators of mutagenesis and genotoxicity in nonsmokers, passive smokers, and smokers. A study of risk factors for acute leukemias in adults has been started in collaboration with Cancer and Leukemia Group B (CALGB), a collaborative cancer treatment group with more than 20 member institutions. This study has an emphasis on identifying risk factors for cytogenetically defined subgroups of acute leukemia and will utilize the results of bone marrow cytogenetics being done as part of CALGB treatment protocols to classify patients into potentially etiologically distinct subgroups. The study is intended to confirm previously reported associations between occupational and environmental exposure to chemicals and risk for specific types of leukemia marked by clonal chromosomal changes. The study will also evaluate leukemia risk from childhood exposure to cigarette smoke. This association was demonstrated in the program's recently completed study of cancer risk from passive smoking. Cigarette smoke exposure, too, may be more strongly associated with specific leukemia subgroups that are defined by cytogenetic abnormalities.

PROJECT DESCRIPTION

METHODS EMPLOYED: (1) A case-control study of adult cancer risk and smoking by spouse or by parents during pregnancy and childhood was conducted using cancer patients from UNC Memorial Hospital and both randomly selected healthy controls and controls identified as friends of cases. Cases and controls were interviewed by telephone to obtain smoking histories of study subjects, their parents, and their spouses. Data on smoke exposure were collected from over 500 cases, 500 controls and also from 700 of their mothers and siblings. Data from mothers and siblings are being used to determine the reliability of parental smoking histories provided by adult offspring, and to develop recommendations for future studies. (2) A cohort of 60,000 individuals for whom information about their own smoking habits and those of household members was obtained in 1963 as part of a population census in western Maryland will be followed through the use of existing records to determine death rates, specific causes of death, and cancer incidence through 1975. Available records include tumor registry entries, death certificates, and other data collected during the 1963 special census. Death rates and cancer incidence rates for adults living with smokers will be compared with those for persons not living with smokers in 1963. Risk associated with exposure to household members who smoke will be evaluated for both active smokers and nonsmokers. (3) A case-control study of incident acute leukemia cases is being conducted in collaboration with Cancer and Leukemia Group B, a cooperative cancer treatment group. All cases eligible for inclusion in a CALGB first line treatment protocol will be asked to participate in an epidemiologic study. Population controls will be selected through random telephone screening. Cases and controls will be interviewed by telephone (cases will be interviewed while they are still hospitalized) to obtain information on environmental and occupational exposures, past disease history, and family history of cancer and other disease. Questionnaires emphasize exposure to chemicals and cigarette smoke. Results of bone marrow cytogenetic studies done by CALGB under other protocols will be used to classify patients by predominant chromosomal abnormality. Cases and controls will be compared to estimate the relative risk of leukemia associated with specific exposures. Exposure histories for specific cytogenetically defined subgroups will be evaluated to determine if certain risk factors are more strongly associated with leukemia in which particular chromosomal changes are apparent. Cases will be enrolled over a three year period. Approximately 250 patients per year (and 250 population controls) are expected. (4) Biochemical markers of genetic damage are being measured in smokers, nonsmokers who live with smokers, and nonsmokers with no smoke exposure. Blood and urine samples are collected from healthy volunteers in each of the three smoke exposure categories. Information on smoke exposure is collected using a short questionnaire. Exposures are validated by measuring cotinine and thiocyanate in blood and urine. Fresh blood samples are used to measure sister chromatid exchanges in lymphocytes and urine samples are being frozen for later use in modified Ames assays for urinary mutagens.

MAJOR FINDINGS AND PROPOSED COURSE: (1) Retrospective study of cancer risk from early life exposure to cigarette smoke has proven to be feasible. Qualitative information provided by study subjects on smoking of parents was accurate, although quantitative data were less reliable. Smoking by fathers was

associated with a fifty percent increase in overall cancer risk and smoking by spouse was associated with a sixty percent increase in risk that was independent of active smoking by the case. Mother's smoking contributed only slightly to overall cancer risk, but few mothers were smokers. Cancer risks associated with exposure to cigarette smoke were elevated for smokers and nonsmokers and not limited to known smoking related sites. Cancer risk also increased with increasing numbers of household members who smoked. Specific sites which warrant further study were identified and will be pursued in other Branch studies. Women married to smokers were also found to have an earlier age at menopause than women married to nonsmokers. Additional analysis of the data will be conducted to further evaluate the quality of the data obtained from subjects about their parent's smoking and recommendations for future studies will be developed. (2) Tumor registry data are being reviewed and abstracted to identify members of the study population who developed cancer between 1963 and 1975. Census and mortality data tapes have been merged, and 9000 deaths from cancer have been identified to date. Data collection and analysis will require an additional 6 months. (3) Study materials such as data collection forms and the questionnaires are being developed. A pilot phase of data collection will be conducted in the Fall of 1985, with full enrollment beginning January 1986. (4) 42 women aged 18-40 were enrolled in the biochemical study. This group includes 15 nonsmokers, 14 passive smokers, 7 moderate smokers, and 6 heavy smokers. Cytogenetic assays (both a standard and a modified assay for sister chromatid exchanges) were successfully performed on 41 of the 42 blood samples. Urine specimens from all participants were frozen and will be analyzed for urinary mutagens. Blood and urine for cotinine and thiocyanate determinations have been sent to the American Health Foundation. Preliminary analysis of the sister chromatid exchange data suggests small differences between smokers and nonsmokers and confirms earlier work suggesting the superiority of the modified assay for detection of small differences due to smoking.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Even though cancer has been intensively studied by epidemiologists, there is clearly room for new approaches, especially approaches that look for biological pathways of carcinogenesis that have not been fully explored (e.g. passive smoking) or that take advantage of rapidly-developing laboratory technology (e.g. chromosomal analysis). The goal of our cancer research is not only to identify specific carcinogens in the environment but to enlarge our understanding of the mechanisms by which long-term, low-level exposure to environmental chemicals can lead to cancer in humans. The successful use of secondhand smoking histories in the study of passive exposure to cigarette smoke may encourage other researchers to pursue this important area of research. Because the study findings include an association of passive smoker exposure and cancer risk at sites not thought to be related to cigarette smoke, reports from this study may encourage other studies. The branch's studies provide support for the notion that passive exposure to sidestream smoke is systemic and may be qualitatively different from direct exposure to mainstream smoke. The study also suggests the need for future studies of risk from smoking in which passive smokers are not included in the control group of nonsmokers.

The study of cytogenetically define subgroups of acute leukemia is a first step in understanding the role played by chromosome abnormalities in the development of the disease. The use of precise subgroups of disease, as in the study of cytogenecity defined subgroups of leukemia, may help to clarify risk factors. Particular risk factors that are associated with only a small subset of cases cannot be identified in studies which combine groups of cases.

PUBLICATIONS

Sandler, D.P., Everson, R.B., Wilcox, A.J.: Passive smoking in adulthood and cancer risk. *Am J Epidemiol.* 121:37-48, 1985.

Sandler, D.P., Wilcox, A.J., Everson, R.B.: Cumulative effects of lifetime passive smoking on cancer risk. *Lancet* 1:312-315, 1985.

Sandler, D.P., Everson, R.B., Wilcox, A.J. Browder, J.P.: Cancer risk in adulthood from early life exposure to parents' smoking. *Am J Public Health* 75:487-492, 1985.

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LAWRENCE LIVERMORE LABORATORY/UNIVERSITY OF CALIFORNIA

1Y01-ES-3-0114-01

Title: Human Studies of Somatic, Germinal, and Heritable Genetic Damage

Contractor's Project Director: Andrew Y. Wyrobek, Ph.D.

Project Officer (NIEHS): Richard B. Everson, M.D., Medical Officer
Epidemiology Branch

Date Contract Initiated: September, 1983

Current Annual Level: \$90,000

PROJECT DESCRIPTION

OBJECTIVES: To investigate the capability of existing and newly developed laboratory approaches to determine the extent and persistence of somatic, germinal, or heritable genetic damage in man and relate these findings to the risk of cancer and other diseases among exposed subjects.

METHODS EMPLOYED: Initial studies will investigate the genetic damage associated with the administration of cytotoxic drugs used for cancer chemotherapy or immunosuppression by laboratory analysis of blood specimens from cohorts of exposed patients. Specimens are being collected from groups of individuals undergoing treatment by selected chemotherapeutic drugs using a longitudinal design, with the same individual sampled before, during, and after treatment. Treatments currently being studied are three combinations of drugs given intravenously to women with breast cancer after resection of all detectable tumor. These combinations are vinblastine, methotrexate and 5-fluorouracil (VMF) cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) and cyclophosphamide, adriamycin, and 5-fluorouracil (CAF). Of the 110,000 women in the United States who develop breast cancer, current trends in cancer treatment suggest that 90 percent will be eligible to receive these or similar drug combinations and about 50 percent will survive 10 years, so that the carcinogenic risk and other effects of these exposures should be well documented. Assays currently being conducted under this agreement include cytogenetic analyses of human lymphocytes for chromosomal aberrations and sister chromatid exchanges.

Later studies will apply these assays to studying effects from less intense or less well defined environmental exposures.

MAJOR FINDINGS AND PROPOSED COURSE: The study design has been completed and enrollment of study subjects has been initiated. Preliminary results demonstrate increased frequencies of chromosomal aberrations and sister chromatid exchanges among blood cells of women being treated with CMF and CAF. The relative persistence of these effects is being characterized. Analysis of blood specimens for glycophorin mutation in red cells, an assay sensitive to specific locus mutations, is planned.

SIGIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Studying effects of cytotoxic drugs provides a potentially efficient means of evaluating such issues as the sensitivity and specificity of human genotoxicity assays. Furthermore, existence of clinical data concerning long term effects should enable comparison of risk with effects in human biomonitoring assays. Since these exposures and assays can be mimicked in experimental animals and in some instances short-term in vitro tests, information generated in this study could provide an important source of data for judging similarities and differences between effects in vivo in humans and in experimental systems.

LABORATORY OF BIOCHEMICAL RISK ANALYSIS

LABORATORY OF BIOCHEMICAL RISK ANALYSIS

In extrapolation from laboratory data to humans, increasing research emphasis has been placed on defining structure-activity relationships for the toxic actions of chemicals. Because of these efforts, the sequence of biochemical events ultimately leading to manifestations of toxicity is becoming more clear for some chemicals. By obtaining a detailed understanding of macromolecular interactions in sensitive and non-sensitive species, predictions concerning human sensitivity might be possible. The use of state-of-the-art mechanistic information, such as dioxin receptor interactions, DNA adduct quantitation and oncogene evaluation could enable us to: remove some of the highly uncertain assumptions that are made in species extrapolation of risk including carcinogenesis; and more accurately predict non-oncogenic effects such as organ-specific toxicity. NIEHS is in a unique position to utilize molecular and toxicokinetic techniques in the characterization of host factors that predispose or protect human populations to environmentally-mediated diseases. The Institute has the capacity to generate appropriate animal models to validate assay methods, and utilize direct collaboration of intramural molecular biologists, pharmacologists, epidemiologists and mathematicians to investigate, in a comprehensive way, the underlying mechanisms responsible for epidemiological observations. Our molecular epidemiology studies attempt to characterize early biochemical indicators predictive of responses to carcinogens and other toxicants at the biochemical target site. Several examples of this approach are documented by the research accomplishments and plans of the Laboratory as indicated in the following paragraphs.

Scientists in the Laboratory of Biochemical Risk Analysis have demonstrated that placentas obtained from women in Taiwan who were accidentally exposed to rice oil contaminated with PCBs had dramatically elevated concentrations of a specific enzyme system (one isozyme of cytochrome P-450) although analyses were conducted four years after the exposure had occurred. Clinical symptomology characteristic of exposure to toxic halogenated aromatics were present in many of the newborns as well as the mothers. Molecular biology studies revealed that increased enzyme activity was a consequence of the induction of a specific protein that is also induced in rabbit lung following exposure to toxic halogenated aromatics. Ongoing studies are evaluating the role of TCDD receptor in individual variation in responsiveness to PCBs as well as the polychlorinated dibenzofurans (PCDFs), and we are measuring concentrations of PCB and PCDF congeners in placenta and blood. These findings will permit the NIEHS to study mechanisms responsible for individual variation in responsiveness to PCBs and related compounds present in the rice oil which may lead to a rational process for identifying groups at risk to the toxic effects of compounds such as PCBs and dioxins.

Cytogenetic studies in the Laboratory of Biochemical Risk Analysis have developed a modified assay that greatly enhances our ability to detect genetic damage from smoking. The assay evaluates sister chromatid exchange (SCE) frequencies in human lymphocytes from smokers or non-smokers following in vitro exposure to α -naphthoflavone (ANF). Although no difference in SCE frequency was detected between smokers and non-smokers in the absence of ANF, in vitro challenge with this chemical produced a large

increase in SCEs in lymphocytes from smokers and only a small increase in samples from non-smokers. All samples from smokers had higher SCE frequency than any sample from non-smokers. Further work is focussing on the mechanisms responsible for ANF-mediated increase in sensitivity of cytogenetic damage in smokers. These studies may allow us to monitor human populations exposed to certain classes of environmental agents for genetic damage prior to the onset of overt toxic syndromes. For example, lymphocytes from the PCB-exposed population in Taiwan are being examined for cytogenetic damage using our modified SCE assay.

There is a burgeoning knowledge related to the critical role of a family of genes in the carcinogenic process. These genes are called oncogenes and they are activated in many kinds of tumor tissue. Utilizing the unique capacity of the NIEHS to investigate carcinogenicity in animal models BRAP and TRTP are developing a research effort aimed at establishing mechanisms to utilize data on oncogene expression in risk analysis of specific classes of chemical carcinogens. Specific aims are: (1) To examine the tissue and chemical selectivity for oncogene activation in chemically-induced tumors derived from the NTP carcinogen bioassay program. (2) To examine oncogene activation in spontaneously arising tumors in animals and to compare with oncogene activation in chemically-induced tumors. (3) To investigate the time-course of oncogene activation and expression in order to establish the earliest time after carcinogen administration at which the transforming genes can be detected. (4) To determine quantitative relationships between receptor occupancy, changes in gene expression and tumor promotion for different classes of promoting agents focussing on TCDD and related compounds and estrogenically-active chemicals. These studies will allow us to address many important public health questions including possible classification of chemical carcinogens according to mechanism of action. Results to date have characterized the properties of activated oncogenes in spontaneous tumors from the B6 mouse, tetranitromethane-induced liver tumors in mice and furfural-induced tumors in rats. Moreover, we have established the rat two-stage model for hepatocarcinogenesis using diethylnitrosamine as the initiating agent and TCDD or 17 α -ethinylestradiol as the promoting agents. These studies have provided data which suggest that TCDD and estrogen receptors in liver are important to the promotion process.

There is considerable controversy regarding the influence of route of exposure of benzene on the toxicity of this compound, particularly its carcinogenic effects. Laboratory scientists, in collaboration with STB and NTP are addressing this question by performing pharmacokinetic studies in animals exposed to benzene either by the oral or inhalation route. These investigations use a wide dose-response range including exposures as low as those encountered in the workplace. Key metabolites are quantified in target tissues and blood and cytogenetic effects are evaluated in relation to the pharmacokinetic and metabolism data. Follow-up studies will determine if DNA adduct concentrations are dose dependent.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 35005-06 LBRA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Carcinogen-Induced DNA Damage and Repair

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Marshall W. Anderson Res. Mathematician LBRA NIEHS

Others: Dr. Felix Romagna Visiting Fellow LBRA NIEHS
Dr. Steve Belinsky NIH Postdoctoral LBRA NIEHS
Ms. Catherine White Bio. Lab. Tech. LBRA NIEHS
Ms. Coleen Hunnicutt Biologist LBRA NIEHS
Dr. Claudia Thompson Staff Fellow LBRA NIEHS

COOPERATING UNITS (if any)

Dr. Richard Philpot and Ms. Teddy Devereux, Laboratory of Pharmacology
Dr. Jim Swenberg, CIIT

LAB/BRANCH

Laboratory of Biochemical Risk Analysis

SECTION

Molecular Toxicology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

1.5

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

There is compelling evidence that many mutagens and carcinogens are able to react with cellular DNA either directly or following metabolic formation of reactive products. If DNA replication proceeds on such a modified template before altered bases or nucleotides are removed by enzymic repair processes, the mutations can be genetically fixed. Thus, the extent of carcinogen-induced promutagenic DNA damage and the capacity of cells to repair such damage represent critical events in the initiation of carcinogenesis. We are studying the in vivo formation and repair of carcinogen metabolite-DNA adducts in tissues and cells that are susceptible or resistant to carcinogen-induced neoplasia. We are concerned with the effects of dose of carcinogen on the amounts and types of adducts formed and on the subsequent repair of these adducts. Our studies on the in vivo formation and repair of benzo(a)-pyrene (BP) metabolite-DNA adducts in a variety of tissues and cell types emphasize the possibility that long-term exposure to low levels of BP could result in the accumulation of BP-DNA adducts in cells which have slow turnover rates. Even if environmental exposure to BP (and other polycyclic aromatic hydrocarbons) is too small to induce neoplasia, the persistence of BP-DNA adducts may produce aberrations in transcripts of genetic information in various cell types and lead to other toxic effects. Studies with 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyridyl)-1-Butanone (NNK), a major nitrosamine formed in tobacco smoke, have demonstrated that site specificity for tissue alkylation and carcinogenesis by NNK are identical. High concentrations of 0.6MG in the lung and nasal cavity may be important factors in the induction of respiratory tumors whereas accumulation of 0.4MdT may be involved in the induction of hepatocellular carcinoma by NNK. DNA repair was examined in isolated lymphocytes after treatment of rats in vivo with various carcinogens. The in vitro lymphocyte system in conjunction with specific inhibitors of DNA repair may be a valuable approach to detect low levels of in vivo carcinogen-induced DNA damage and to study the mechanism of excision repair.

PROJECT DESCRIPTION

OBJECTIVES AND METHODS EMPLOYED:

1. We will continue to examine the in vivo formation and subsequent repair of carcinogen-DNA adducts in both target and non-target cells. Several hypotheses will be further tested: a) Does initial adduct levels and their persistence in target cells contribute an effective measure of the biological dose of the carcinogen, and b) does carcinogen-induced DNA damage in non-target cells impose an additional toxicological risk.
2. We will examine possible interactions between carcinogens and the damage they inflict on DNA. We will test the hypothesis that DNA damage induced by one carcinogen inhibits the repair of damage induced by another carcinogen.
3. To continue to explore the mechanism(s) by which BHA and aryl hydrocarbon hydroxylase (AHH) inducers inhibit carcinogen-induced neoplasia. The relationship between inhibition of BP metabolite-DNA adduct formation and induction or repression of the synthesis of specific forms of cytochrome P-450 will be examined as a function of time after TCDD or BHA administration. This will be the first step in testing the hypothesis that changes in the concentrations of specific forms of cytochrome P-450 are related to the anticarcinogenesis mode of action of AHH inducers or BHA.
4. To develop in vitro system to examine repair of chemical-induced DNA damage in vivo. The feasibility of using isolated lymphocytes will be explored.

The experimental approach used to investigate these objectives is as follows:

1. Formation and persistence of carcinogen-DNA adducts: Animals were treated with various doses of carcinogen and then sacrificed at relevant time points. Radiolabeled carcinogen was used in some studies. Specific cell types were isolated from liver (hepatocytes and non-parenchymal cells) and lung (type II, Clara, and macrophages). Respiratory mucosa was obtained from the naso- and maxilloturbinates, lateral walls, and medial septum anterior to the olfactory epithelium. DNA was isolated from tissues or cells by pronase and RNase digestion, phenolic extraction and ethanol precipitation. Isolated DNA was either enzymatically digested to individual nucleosides or depurinated by acid and heat treatment. High pressure liquid chromatography (HPLC) procedures were developed to analyze for carcinogen metabolite - DNA adducts. A competitive radioimmune assay was employed for the measurement of O⁴-methyldeoxythymidine and O⁶-methyldeoxyvanine, ³²P-postlabeling is being used to examine the bulky DNA adducts formed with NNK.
2. Examination of DNA repair processes: Carcinogen-induced DNA repair in lymphocytes was examined by unscheduled DNA synthesis (UDS) measurements or by nucleoid sedimentation assay. After treatment of animals with carcinogens or vehicle, lymphocytes were isolated, incubated in vitro and

DNA repair measured throughout the incubation period. UDS was examined by separating de novo DNA synthesis from repair synthesis by alkaline CsCl gradient(s). Cells were prelabeled with BRDU to density label the replicating DNA. UDS is measured in non-replicated DNA by ³H-cytidine incorporation. In the nucleoid assay system, cells are homogenized in a solution containing 2-mercaptoethanol and nucleoids prepared with a modified procedure previously developed by Cook and Brazell. The sedimentation of the nucleoids are examined in neutral sucrose gradients. Changes in sedimentation distances reflect changes in superhelicity induced by strand breaks occurring during excision repair.

MAJOR FINDINGS AND PROPOSED COURSE:

1. We have characterized the in vivo formation of BP metabolite-DNA adducts in a variety of target and non-target tissues of mice and rabbits. Tissues included were lung, liver, forestomach, colon, kidney, muscle, and brain. The major adduct identified in each tissue was the (+)-7 β ,8 α -dihydroxy-9 α -epoxy-7,8,9,10-tetrahydro-BP (BPDEI)-deoxyguanosine adduct. A 7 β ,8 α -dihydroxy-9 β , 10 β -epoxy-7,8,9,10-tetrahydro-BP (BPDEII)-deoxyguanosine adduct, a (-)-BPDEI-deoxyguanosine adduct, and an unidentified adduct were also observed. The adduct levels are unexpectedly similar in all the tissues examined from the same BP-treated animal. For example, the BPDEI-DNA adduct levels in muscle and brain of mice were approximately 50% of those in lung and liver at each oral BP dose used. We have also examined adduct levels formed in vivo in several cell types of lung and liver. Macrophages, type II cells, and Clara cells from lung and hepatocytes and nonparenchymal cells from liver were isolated from BP-treated animals. BPDEI-deoxyguanosine adduct was observed in each cell type and, moreover, the levels were similar in various cell types. There are several explanations for the in vivo binding of BP metabolites to DNA of most tissues (cells). It is possible that the oxidative metabolism of BP in each of the examined tissues is sufficient to account for the observed DNA binding. However, there is obviously no correlation between monooxygenase activity and DNA binding. It is also possible that BP is metabolized in tissues such as lung and liver and then BPDEI and II are transported to tissues such as brain and muscle where they bind to DNA. In any case, exposure of animals to BP results in the binding of BP metabolites to the DNA of all tissues examined and moreover, the relative binding levels among the tissues in the same BP-exposed animals are similar. Since this observation is true for each animal species examined, for each BP dose used and for each route of administration employed, and since in vitro incubation of human tissue with BP results in the formation of BPDE-DNA adducts, it is very probable that DNA in many human tissues is continuously damaged by exposure to this ubiquitous environmental carcinogen and other PAHs.
2. These results on adduct formation and other findings from our laboratory on persistence of adducts emphasize the possibility that long-term exposure to even low levels of BP could result in accumulation of PAH metabolite-DNA adducts in cells which have slow turnover rates. Even if environmental exposure to PAH is too small to induce neoplasia, the persistence of DNA

adducts may produce aberrations in transcripts of genetic information in various organs and lead to other toxic effects.

3. 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyridyl)-1-Butanone (NNK), a major nitrosamine formed in tobacco smoke, induces a high incidence of lung, liver and nasal cavity tumors in rats. Since α -hydroxylation of NNK by target tissues can lead to the generation of a methylating agent, the formation and removal of the promutagenic lesions, O⁶methylguanine (O⁶MG) and O⁴-methyldeoxythymidine (O⁴MedT), were determined over 12 days of NNK administration to rats (100 mg/kg/day). The concentration of O⁶MG increased steadily in lung throughout the treatment regimen, while O⁶-methylguanine-DNA methyltransferase (O⁶MGMT) decreased to less than 5% of control. The concentration of O⁴MedT in lung DNA reached a steady state after 4 days of carcinogen treatment. After NNK treatment was discontinued, O⁶MG persisted while O⁴MedT was removed rapidly in the lung, suggesting the different repair pathways exist for the removal of these adducts in vivo. In hepatocytes, nonparenchymal cells and nasal mucosa, O⁶MG concentrations were maximal after 2 days and declined by 50-80% during the remaining 10 days of treatment. The decrease in O⁶MG levels in nasal mucosa paralleled a decline in O⁶MGMT activity and was associated with marked cytotoxicity to Bowman's glands, portions of the lateral nasal gland, and the olfactory and respiratory mucosa during carcinogen treatment. In contrast, the decline in DNA alkylation in hepatocytes was attributed to the induction of O⁶MGMT activity. These experiments have demonstrated that site specificity for tissue alkylation and carcinogenesis by NNK are identical. The accumulation and persistence of methylated DNA adducts observed during repeated exposure to NNK could be a major contributing factor to the strong association between tobacco use and cancers of the respiratory system.
4. We have begun to examine lymphocytes as a model to study repair of DNA lesions induced in vivo. It was shown that isolated lymphocytes from rats have the capacity to repair in vitro DNA damage induced in vivo by several carcinogens (methyl-nitrosurea and 4-nitroquinoline-1-oxide). Moreover, the in vitro repair rates corresponded to those measured in vivo. DNA repair was measured by the nucleoid sedimentation assay and unscheduled DNA synthesis (UDS). Repair-induced DNA strand breaks could be accumulated in the presence of the polymerase α -inhibitor aphidicolin. This allowed us to detect DNA damage induced by relatively low doses of these carcinogens. The sensitivity to detect strand break was increased by at least a factor of 10. The effect of aphidicolin was reversible since the partially inhibited excision repair process was completed after removal of the inhibitor from the incubation medium. Also, aphidicolin had very little effect on UDS whereas semi-conservative DNA synthesis was completely inhibited. This observation needs further clarification in order to use this inhibitor as a tool to simplify UDS measurements. The use of the in vitro lymphocyte system in conjunction with specific inhibitors of DNA repair may be a useful approach to detect low levels of in vivo carcinogen-induced DNA damage and to study the mechanism of excision repair. Of course, lymphocyte DNA will not be damaged from exposure to all types of chemicals and it will be important to determine the classes of chemical for which lymphocytes will be a good model to assess DNA damage and repair.

Plans for the subsequent year are as follows:

1. To examine the formation of methylated DNA adducts formed by NNK and to study the repair of these adducts in specific cell types of lung and nasal mucosa. To examine cell specific toxicity in lung and nasal cavity epithelium. Some emphasis will be placed on the characterization of the bulky adducts formed by NNK and NNN. These studies should aid in the extrapolation of high-dose toxicity data to low exposure doses of these tobacco specific carcinogens.
2. To examine the effects of AHH inducer, i.e., TCDD, on the formation of NNK metabolite-DNA adducts in lung, liver, and nasal mucosa of rats. These are the target tissues for NNK-induced neoplasia in rats. AHH inducers are known to dramatically inhibit both BP-induced neoplasia and BP metabolite-DNA adducts in mice. The effects of AHH inducer on NNK-induced neoplasia or NNK-DNA adducts formation has not been examined. These studies will compare the effects of AHH inducers on DNA adducts formed from representative members of two classes of carcinogens, polycyclic aromatic hydrocarbons and nitrosamines, in the same model system.
3. To continue to characterize carcinogen-induced DNA repair in lymphocytes. Repair will be examined as a function of amounts of adducts formed initially and as a function of time after initial DNA damage. DNA repair will also be examined after lymphocytes are stimulated to proliferate. Further attempts will be made to increase the sensitivity to measure DNA repair by utilization of inhibitors of specific steps in the excision repair process (See #4 in previous section).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: There is compelling evidence that many mutagens and carcinogens are able to react with cellular DNA either directly or following metabolic formation of reactive adducts. If DNA replication proceeds on such a modified template before altered bases or nucleotides are removed by enzymic repair processes, the mutations can be genetically fixed. Thus, the extent of carcinogen-induced promutagenic DNA damage and the capacity of cells to repair such damage represent critical events in the inhibition of carcinogenesis. Although there have been numerous studies of chemical-induced DNA damage and repair processes in in vitro model systems, in vivo studies have been neglected. An analysis of the factors that determine the initial extent of in vivo carcinogen-DNA adduct formation and the capacities of the various repair processes in target and nontarget tissues (cells) should provide relevant information about tissue and cellular selectivity in chemical-mediated neoplasia and other toxicological responses.

A problem of practical importance in environmental toxicology is to predict the potential chemical insult to man from high dose toxicology data in laboratory animals. Thus, both low dose and species-to-species extrapolation of toxicology data are involved in the risk assessment of human exposure to chemicals. Carcinogen-DNA adduct levels and repair rates of the adducts can usually be measured at much lower doses of the carcinogens than the doses used in bioassay studies. The potential use of the amount and persistence of carcinogen-DNA

adducts formed in the target cell as a measure of the effective dose of a carcinogen should help in the low dose and species-to-species extrapolation of carcinogenic data.

PUBLICATIONS

Kulkarni, M. and Anderson, M. W.: Persistence of benzo(a)pyrene metabolite-DNA adducts in lung and liver of mice. *Cancer Res.* 44: 97-101, 1984.

Kulkarni, M., Angerman-Stewart, J., and Anderson, M. W.: Detection of in vivo DNA repair synthesis in mouse liver and liver induced by treatment with benzo(a)pyrene or 4-nitroquinoline-1-oxide. *Cancer Res.* 44: 1547-1550, 1984.

Stowers, J. S. and Anderson, M. W.: Ubiquitous binding of benzo(a)pyrene metabolites to DNA and protein in tissues of mouse and rabbit. *Chem. Biol. Interact.* 51: 151-166, 1984.

Romagna, F., Kulkarni, M., and Anderson, M. W.: Detection of repair of chemical-induced DNA damage in vivo by the nucleoid sedimentation assay. *Biochem. Biophys. Res. Commun.* 127: 56-62, 1985.

Belinsky, S. A., White, C. M., Boucheron, J. A., Richardson, F. C., Swenberg, J. A., and Anderson, M. W.: Accumulation and persistence of DNA adducts in respiratory tissue following multiple administration of the tobacco specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1 butanone. *Cancer Research* (submitted).

Horton, J. D., Rosenoir, J., Bend, J. R., and Anderson, M. W.: Quantitation of benzo(a)pyrene metabolite-DNA adducts in selected hepatic and pulmonary cell types isolated from ³H-benzo(a)pyrene-treated animals. *Cancer Res.* (in press).

Anderson, M.W., Adriaenssens, P.I., White, C.M., Ioannou, Y.M., and Wilson, A.G.E.: Effect of the antioxidant BHA on in vivo formation of BP metabolite-DNA adducts; Xenobiotic Metabolism: Nutritional Effects, ACS Symposium Series No. 277, 241-251, 1985.

Stowers, J.S. and Anderson, M.W.: Formation and persistence of benzo(a)pyrene metabolite-DNA adducts. *Environ. Health Perspect.* (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 46003-01 LBRA		
PERIOD COVERED October 1, 1984 to September 30, 1985				
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Lymphocyte markers for evaluating exposure and biologically-effective dose				
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)				
PI:	Claudia Thompson	Staff Fellow	LBRA	NIEHS
	George Lucier	Chief	LBRA	NIEHS
Others:	K. Lundgren	Visiting Fellow	LBRA	NIEHS
	M. Andries	Visiting Fellow	LBRA	NIEHS
	M. Anderson	Res. Mathematician	LBRA	NIEHS
	I. Zajac	Chemist	LBRA	NIEHS
	O. McDaniel	Bio. Lab. Tech.	LBRA	NIEHS
	J. Lambert	Bio. Lab. Tech.	LBRA	NIEHS
COOPERATING UNITS (if any) Epidemiology Branch, BRAP Preventive Medicine Institute - Strang Clinic New York City, NY				
LAB/BRANCH Laboratory of Biochemical Risk Analysis				
SECTION Cellular Epidemiology				
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709				
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:		
6	3	3		
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews				
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>It is the long-range plan of this project to evaluate the relationship between the "biologically-effective dose" for a particular chemical and the effects of prolonged or acute exposure on cellular processes that may be important in tumorigenesis. Animal models and defined human populations exposed to environmental substances will be used to evaluate the quantitative relationship between exposure dose and DNA-adduct levels; to determine the utility of lymphocytes as a molecular dosimeter of environmental exposure; to identify biochemical, molecular and cytogenetic markers to distinguish chemically-exposed individuals from nonexposed; and to evaluate the role of genetic factors in modulating cellular processes that influence the effect of a given exposure. Currently, a procedure has been developed that distinguishes smokers from nonsmokers, and methods are being developed to quantitate DNA adducts in exposed individuals by post-labeling. Individuals have been identified that have been exposed to substances of interest and biochemical assays are developed to determine the relationship of adducts and exposure to cellular processes related to DNA damage and repair. The goals of these studies are to understand some of the basic biochemical systems important to genetic damage in human populations and to apply the knowledge obtained to identify individuals that may be at increased risk to develop cancer in later life.</p>				

PROJECT DESCRIPTION

OBJECTIVES AND METHODS EMPLOYED: It is the objective of this project to use lymphocytes as a biologic tool to:

1. Determine the "biologically-effective dose" for a chemical carcinogen by evaluating the quantitative relationship between exposure dose and DNA-adduct levels in target tissues and lymphocytes following different routes of exposure.
2. Identify biochemical, molecular and/or cytogenetic markers that distinguish chemically-exposed individuals from unexposed individuals and/or individuals genetically predisposed to develop cancer from those that are not predisposed.
3. Determine the effects of prolonged or acute exposure to environmental substances on cellular processes involved in metabolic activation/deactivation pathways, DNA binding and defined human aliphoid sequences and DNA repair
4. Assess the role of genetic factors in modulating cellular processes that influence the effect of a given exposure.

Measurement of human exposure based on ambient levels of substances in the air or water do not accurately measure individual exposure. Although measurement of serum or urine levels of a substance does give an indication of individual burden, it may not be a good indicator of biological effects of the exposure. The pharmacokinetics of cellular uptake, metabolism and excretion of a substance may vary considerably from person-to-person so that biological effects at a given level of exposure will also vary. It is widely believed that DNA damage is a necessary step in the process of tumorigenesis. Therefore, measurement of the level of DNA damage is likely to provide a better indicator of biological effects.

Post-labeling methods have been developed that combine the technique of in vitro labeling of nucleotides derived from cellular DNA with ³²phosphorous and the methods of high pressure liquid chromatography (HPLC) or thin layer chromatography (TLC) to separate the labeled nucleotides. The primary application of the post-labeling method will be to determine the "biologically-effective dose" for different classes of chemical carcinogens. Results from animal studies will provide accurate molecular dosimetric relationships between exposure level, route of exposure and DNA-adduct levels. Moreover, by comparing DNA-adduct levels in target organs and in lymphocytes we will be able to evaluate the usefulness of DNA isolated from lymphocytes as a molecular dosimeter of exposure in major organ systems. We will first apply the post-labeling technique to model chemicals for which DNA adducts have been well characterized and then apply this technology to environmental or occupational substances which have been less well-characterized to determine the facility of this technique to general environmental toxicology.

Sprague-Dawley rats will be exposed to varying doses of model compounds such as benzo(a)pyrene and acetylaminofluorene by inhalation, oral or subcutaneous injection administrations. Both radioactive and nonradioactive compounds will be used for injections and oral administration so that the accuracy and efficiency of post-labeling of modified nucleotides can be assessed. An advantage of the post-labeling assay is that previously low dose effects had to be extrapolated from high dose data. With the increased sensitivity of ^{32}P post-labeling, lower doses can be administered for detection of adducts that were below the level of sensitivity of existing assays. DNA will be isolated from lymphocytes and several organ systems at various times following or during the exposure regiment. DNA-adducts will be quantitated by measuring either the amount of $[\text{}^3\text{H-}]$ DNA adducts or $[\text{}^{32}\text{P}]$ DNA adducts. The persistence of DNA adducts in target tissues and circulating lymphocytes will be determined by measuring levels at various times following the end of the exposure. Moreover, urine and blood samples will be collected to measure the level of the carcinogen to determine body burden during and following exposure. These values will be used to evaluate the relationships between body burden and adduct levels.

Once the molecular dosimetry for known carcinogens has been characterized by post-labeling methods, the application of this technology to environmental or occupational substances, for which biological effects are less clearly understood, will be pursued using similar experimental procedures. Studies will also be undertaken whereby individuals will be identified that have been occupationally or environmentally exposed to toxic substances that have been extensively studied in animals. Blood and urine will be collected to measure body burden and DNA-adduct levels quantitated in lymphocytes to determine whether information obtained from animal studies can be extrapolated to the human situation.

Presently, the level of detection of DNA adducts by the post-labeling method is one modified nucleotide per $10^6 - 10^8$ nucleotides. Chronic low level environmental exposures would more likely result in adduct levels lower than the present limits of detection. Accordingly, one of the objectives of the present study will be to increase the sensitivity of the post-labeling assay. Methods for increasing the sensitivity include purifying the adduct prior to post-labeling or adjusting the assay to use larger quantities of DNA.

The detection of DNA adducts does indicate exposure independent of pharmacokinetic variables, however, it provides no information on the biological consequences of such prolonged or acute exposure. Another objective of this project is to better understand the significance of exposure on cellular processes, therefore, biochemical and cytogenetic markers need to be identified which distinguish exposed individuals from unexposed individuals. Once these markers have been identified experimental procedures can be undertaken to elucidate the mechanisms involved in these differences.

Cytogenetic assays such as measuring sister chromatid exchanges (SCEs) have been widely used to (a) determine genotoxic potential for different chemicals and (b) detect genetic damage that may have accumulated during chronic chemical exposure in individuals. Cigarette smoking is a widespread environmental health problem and constituents of cigarette smoke are known carcinogens. Smoking has

been strongly associated with cancer of the lung, oral cavity, esophagus, pharynx, bladder and pancreas. Conflicting results have been reported on the effect of smoking on SCE induction. Recently, a method has been developed in our laboratory that clearly distinguishes smokers from nonsmokers on the basis of induced SCE frequencies following *in vitro* challenge of lymphocytes with α -naphthoflavone (ANF). Interestingly, ANF has never been reported to produce genetic damage. This finding is important because it suggests that chronic exposure to some constituent of cigarette smoke alters biochemical or genetic processes such that subsequent exposures to other chemicals could result in enhanced genetic damage.

Cigarette smoke constituents can directly bind DNA as well as modify the metabolizing enzymes necessary for activation/deactivation of xenobiotics. The mechanism for ANF-induced SCEs could be a result of (1) metabolic alterations in activation/deactivation pathways for ANF and/or (2) inherent genetic instability resulting from cigarette smoking. Several experimental approaches will be undertaken to elucidate the mechanism of ANF induction of SCEs.

1. Lymphocytes will be isolated from smokers and non-smokers and placed in culture. The cells will be challenged with [3 H] BP or [3 H] ANF and the metabolites produced analyzed by HPLC. Both water soluble and insoluble metabolites will be analyzed to determine the effect of smoking on metabolic activation/deactivation pathways. If metabolic factors are involved, the induction of specific P-450 isozymes by smoking will be analyzed by use of monoclonal antibodies and western blots (in collaboration with R. Philpot, LP)
2. In other sets of experiments, lymphocytes will be incubated with the ultimate carcinogenic metabolite of BP, benzo(a)pyrene-7,8-diol,9-10 epoxide (BPDE). The concentration dependent induction of SCEs in lymphocytes of smokers and nonsmokers will then be evaluated. If differences are observed in the frequency of induced SCEs with BPDE, these findings would suggest that smoking produced genetic instability.
3. The major metabolite of ANF is the 5,6-epoxide. Epoxides react readily with DNA, therefore this metabolite will be tested in the SCE assay to determine whether it is capable of inducing SCEs in smokers and/or nonsmokers similar to that seen for BPDE.
4. There are several biochemical assays which may provide good markers for distinguishing smokers from nonsmokers and also provide information on the mechanism for the smoking enhanced induction of SCEs by ANF. Nucleoid sedimentation assays detect DNA single strand breaks by measuring changes in sedimentation of nucleoids through sucrose gradients. Unscheduled DNA synthesis is an assay which measures the induction of DNA repair following damage. Lymphocytes from smokers and nonsmokers will be incubated with ANF or the 5,6-epoxide of ANF. These assays will be performed to determine whether ANF itself causes DNA damage and if there is a difference in the amount or repair of this damage between smokers and nonsmokers.

In addition to identifying cytogenetic and biochemical markers distinguishing smokers from nonsmokers, the consequence of prolonged smoking on cellular processes involved in DNA damage and repair are important to evaluate. Lymphocytes will be isolated from smokers and nonsmokers and incubated with [^3H] BP, [^3H] ANF or other classes of chemical carcinogens. DNA will be isolated and the quantitation, identification and persistence of DNA adducts compared in these two groups by HPLC analysis of deoxyribonucleosides. DNA adducts will be measured in both total genomic DNA and defined aliphoid sequences.

Determination of DNA-adducts in total DNA does not provide information on the effect of DNA-modification at the molecular level. Using the aliphoid sequence as an analytical tool for detection of DNA damage in intact lymphocytes the distribution and effect of damage can be assessed at the molecular level and should provide a better way to correlate DNA adducts with genetic effects. Human genomic DNA contains the aliphoid sequence which is a highly reiterated sequence present in about 300,000 copies per haploid genome. The basic length is 342 nucleotides and the sequence has been determined. Intact lymphocytes from smokers and nonsmokers will be incubated with a carcinogen, the aliphoid sequence isolated and the sequence specificity of modification determined by Maxam-Gilbert sequencing. At the molecular level the results will establish whether smoking changes the sensitivity of the DNA to damaging agents. Moreover, previous reports have indicated that the conformation of the alpha sequence slows down DNA repair of bulky adducts. However, if the conformation of the DNA is altered due to prior exposure of another agent, repair rates were increased. By using the aliphoid sequence the effect of smoking on the conformation of DNA can be evaluated by comparing rates of repair of damage in this sequence between smokers and nonsmokers. These repair rates will also be compared between total DNA and aliphoid sequences.

In conjunction with the human studies to be performed, animal model systems will be developed to mimic the effects observed in smokers. Various constituents of cigarette smoke will be administered to rats by inhalation and the SCE assay developed in our laboratory performed, to elucidate the constituent(s) that are of biological interest. These animals will then be exposed to other carcinogens and the effect on cellular processes determined by the methods outlined. It is the objective of these animal studies to define the biochemical processes which may play important roles in tumorigenesis.

Another cytogenetic marker that will be used in the laboratory is an assay to detect micronuclei. Micronuclei is a cytogenetic marker that reflects genetic damage in lymphocytes following chemical exposure. The chemical benzene is a widespread industrially used compound and may pose a significant health hazard. Micronuclei formation is a sensitive indicator of benzene exposure in animal systems. Recent studies have suggested that benzene or one of its metabolites binds DNA. In collaboration with Lovelace Inhalation animal studies are evaluating dose-dependent aspects of benzene toxicokinetics. In our studies micronuclei and SCE measurements will be used as a biological marker of toxicity and post-labeling techniques will be utilized to quantitate and identify DNA-benzene adducts. The relationship between micronuclei formation and DNA-benzene adduct levels will be evaluated.

Cancer susceptibility cannot in most cases be based solely on environmental factors but the relationship between environment and individual genotype. To assess the role genetic factors play in modulating cellular processes that influence the effect of a given exposure, it would be useful to identify individuals genetically susceptible to developing cancer. Familial polyposis is an autosomal dominant inherited trait that results in an increased susceptibility to developing colorectal cancer. Reported studies have suggested that the DNA repair capacity of these individuals is impaired. Nucleoid sedimentation and unscheduled DNA assays will be performed on lymphocytes isolated from familial polyposis-afflicted individuals and appropriately matched controls. Different classes of chemical carcinogens will be incubated with the lymphocytes to determine whether these assays are sensitive enough to distinguish genetically predisposed individuals from controls.

Induction of xenobiotic metabolizing enzymes are under genetic control. The enzyme glutathione S-transferase is an important enzyme in the detoxification of hazardous chemicals. Individuals can be classified into categories of being either low, moderate or high glutathione conjugators when stilbene oxide is used as a substrate. The role that this enzyme may play in influencing genetic damage will be evaluated by methods described and it's relationship to tumorigenesis evaluated.

MAJOR FINDINGS AND PROPOSED COURSE OF STUDY:

- A. The post-labeling method is presently being developed in the laboratory. The enzymatic reactions for transfer of the δ -phosphate from [^{32}P] ATP to 3'-deoxyribonucleotides has been optimized for normal deoxyribonucleotides. The kinetic's for the reaction have also been determined. HPLC conditions have been developed to separate normal nucleotides from BPDE modified nucleotides. The efficiency of post-labeling BPDE modified nucleotides is presently being evaluated by using calf thymus DNA modified with [^3H] BPDE, where adduct levels are known.

The proposed course of study for the next fiscal year will include developing the TLC system for separation and quantitation of DNA adducts and comparing it with the HPLC method. Optimization of conditions for labeling and quantitating adducts produced from different chemicals will be established so that accurate quantitation of adducts can be achieved. Once the post-labeling method has been clearly defined it will be applied to determine molecular dosimetry using animal studies described in the objectives.

- B. An assay has been developed that differentiates smokers from nonsmokers on the basis of induced SCE frequencies, following in vitro challenge with ANF. It was demonstrated that ANF produced a concentration-dependent increase in the frequency of SCEs in smoking individuals. In preliminary studies, average induced SCE levels were 54% and 13% above baseline levels for smokers and nonsmokers, respectively. The ANF-inhanced increase in the SCE frequency ranged from 3.12 to 5.72 among smokers, and from 0 to 1.96 among the nonsmokers.

In a follow-up study, a larger sample size was used and a dosimetry component was included to investigate the relationship of smoking dose on SCE frequencies. Individuals were grouped into four categories; nonsmokers; passive exposure, moderate (1-15 cigarettes/day) smokers and heavy (16 + cigarettes/day) smokers. Using ANF-induced SCE frequencies as an endpoint, a clear relationship was demonstrated between the smoking dose and the frequency of induced SCEs; SCE frequencies clustered within specific ranges which clearly distinguished nonsmokers, moderate smokers and heavy smokers from each other. Serum was saved from each individual. Cotinine and thiocyanate concentrations will be measured for each individual as an indicator of individual body burden. These values will be used to determine the relationship between body burden and the ANF induced increase in SCE frequency. Also, urine samples will be evaluated for the presence of mutagens by the Ames assay.

The applicability of the SCE assay developed in the laboratory to other potential hazardous chemicals that may act through a similar mechanism is presently being evaluated. High and low dose human exposures to polychlorinated biphenyls (PCBs) have occurred in recent years. For example, in Taiwan rice oil was contaminated with PCB and a number of persons were exposed. Acute toxicity symptoms were diagnosed and long term effects are presently under investigation. Contaminants and by products of PCBs such as polychlorinated quaterphenyls (PCQ) and polychlorinated dibenzofurans (PCDF) may contribute to the toxicity. The PCDFs are considerably more toxic than PCBs and are probably the causative agent in both the Taiwan and Yusho PCB poisoning episodes. PCDFs, like some constituents of cigarette smoke, induce aryl hydrocarbon hydroxylase through a mechanism involving the TCDD receptor.

Taiwanese women exposed to PCBs have been previously identified by scientists in the Epidemiology Branch in a collaborative effort with the Taiwan National Health Department. Blood samples are presently being collected and the ANF-enhanced SCE assay performed. Serum is being collected to measure PCB and PCDF concentrations. Attempts will be made to correlate the concentration of PCDF with ANF-induced SCEs. If the results are similar to that seen for cigarette smokers, animal systems will be developed to determine if the mechanism of ANF induction in PCB exposed individuals operates through a similar mechanism to that of smokers.

- C. Experiments are being conducted to measure metabolism of polycyclic aromatic hydrocarbons in cultured lymphocytes. HPLC conditions have been determined to separate BP metabolites. [³H] ANF is currently being synthesized so that metabolism can be evaluated in smokers and nonsmokers. In collaboration with the Epidemiology Branch, smokers and nonsmokers are being enrolled to investigate the mechanism of ANF-induced SCEs.

The proposed course of study for the next fiscal year will be to pursue the objectives listed in Methods. Metabolism of ANF and BP will be compared in smokers and nonsmokers. The genetic stability will be determined by determining the effects of BPDE on the induction of SCEs. Nucleoid sedimentation and unscheduled DNA synthesis will be assayed in lymphocytes following exposure to ANF and other classes of chemical carcinogens.

- D. Procedures have been developed for purifying DNA from lymphocytes. Restriction digestion of genomic DNA with the endonuclease EcoR, have been optimized and the aliphoid sequence has been purified from genomic DNA by separation on 2% agarose gels and electroelution. End-labeling of the alpha sequence has been achieved by exchange reactions with polynucleotide kinase, and the methods are presently being developed to separate the complimentary strands by polyacrylamide gel electrophoresis. The purposed course of study for the next fiscal year is to improve the purification procedures to increase the yield of the aliphoid sequence. When the experimental methods have been optimized, lymphocytes from smokers and non-smokers will be incubated with [^3H] BP and [^3H] ANF and DNA-adduct levels and sequence specificity of modification determined by HPLC analysis of nucleosides in total DNA and by Moxam-Gilbert sequencing of alpha sequences.
- E. A collaboration has been developed with Dr. Ron Pero of the PMI-Strang Clinic to apply methodologies used for assessing DNA damage and repair rates to identify individuals genetically predisposed towards developing colorectal cancer. Lymphocytes have been isolated from individuals diagnosed as having familial polyposis and from appropriately matched controls.

The proposed course of study for the next fiscal year is to expose lymphocytes isolated from these individuals to different classes of chemical carcinogens and DNA damaging agents. DNA damage and repair will be analyzed by nucleoid sedimentation and/or unscheduled DNA synthesis to determine if there are biochemical differences that may distinguish these populations.

- F. The assay for quantitating and identifying micronuclei in blood is presently being developed. Animal studies and exposure regimens for studying benzene toxicity have been developed. Lovelace Inhalation Facility is dosing the animals by inhalation and oral routes, using radioactive or nonradioactive benzene.

The proposed course of study will be to isolate DNA from target organs and lymphocytes to detect benzene DNA adducts by post-labeling methods. Adducts levels will be compared in different tissues following different routes of exposure, for varying doses of benzene.

SIGNIFICANCE OF BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The human health risks from exposure to toxic environmental agents is becoming increasingly evident. However, conventional epidemiology methods are limited in

their ability to accurately monitor exposure and the immediate biochemical consequences of those exposures. Occupational or environmental exposures occur over long periods of time and to low level of complex mixtures of chemical substances. Much of the evidence accumulated about the etiology of neoplastic disease points to strong influences of environmental factors. It should be considered, however, that the concept of cancer susceptibility cannot in most cases be based solely on environmental factors but the relationship between environment and individual phenotype. The pharmacokinetics of cellular uptake, metabolism and excretion of chemical substances exhibits considerable interindividual variation. Biological indicators of exposure which are independent of pharmacokinetic parameters will be important to determine the "biologically-effective dose" of a compound and to ascertain the consequences of the exposure. It is widely held that DNA damage is a necessary step in the process of tumorigenesis and therefore measurements of the level of DNA damage are likely to provide a better indicator of biological effects. Quantitation of DNA-adducts has been used as one parameter to assess exposure. The recent development of methods to quantitate DNA adducts in the range of 1 adduct in 10^8 nucleotides or less provides the means of evaluating the biological effects of low level exposure which had not been feasible with the technology available. The consequences of the initial damage can manifest itself in a cascade of biological and biochemical events which may eventually lead to neoplasia. The research goals outlined for this project will evaluate the relationship between the "biologically-effective dose" for a particular chemical, the effects of prolonged or acute exposure on cellular processes that may be important in tumorigenesis and the role genetic factors may play in modulating this relationship. The study of chemically-mediated genetic damage in human lymphocytes is the most feasible way of addressing these questions because it is possible to evaluate in vivo as well as in vitro alterations in cell function.

By using animal model systems, methods will be developed to accurately determine molecular dosimetry for different substances which pose potential health risks. This technology can be applied to human populations exposed to environmental agents to determine whether exposure has resulted in biological damage detectable at the genetic level. Biochemical and cytogenetic markers will be identified which distinguishes exposed or genetically susceptible individuals from controls, and the biochemical understanding of these phenomena can be analyzed by using animal models. Through biochemical applications in humans and animals, results obtained should provide basic information on the biochemical systems that may be important in tumorigenesis.

PUBLICATIONS

Lundgren, K. and Lucier, G.W.: Differential enhancement of sister chromatid exchange frequencies by α -naphthoflavone in cultured lymphocytes from smokers and nonsmokers. Mutat. Res. (in press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES-46004-01 LBRA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Receptor Action and Liver Tumor Promotion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation)

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COOPERATING UNITS (if any)

Comparative Pathology Branch, Systemic Toxicology Branch, TRTP, NIEHS,
Statistics and Biomathematics Branch, BRAP, NIEHS

LAB/BRANCH

Laboratory of Biochemical Risk Analysis

SECTION

Receptor Mechanisms Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

5.3

PROFESSIONAL

2.5

OTHER

2.8

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

It is the long range plan of this project to evaluate actions of receptors for toxic halogenated aromatics and estrogenically-active chemicals in relation to hepatotoxic potency of these compounds. These studies focus on receptor mediated effects on gene expression critical to tumor promotion using the rat two-stage model for hepatocarcinogenesis. The compounds of special interest are 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), structurally-related polychlorinated dibenzodioxins and dibenzofurans, diethylstilbestrol, 17 α -ethinylestradiol and γ -zearalanol. The objectives of these studies are to evaluate the quantitative relationships between dose of tumor promoter, receptor occupancy, critical changes in gene expression and histopathological alterations including preneoplastic lesions and tumor incidence. Furthermore, the time course of these changes are being investigated.

PROJECT DESCRIPTION

OBJECTIVES AND METHODS EMPLOYED:

I. Establish the two-stage model for rat liver carcinogenesis in ovariectomized rats using diethylnitrosamine as the initiating agent (single dose) and promoting agents (chronic exposure) that bind either the TCDD receptor or estrogen receptor. Phenobarbital is included to represent a class of liver tumor promoters that has no known receptor. Preneoplastic foci (gamma glutamyltranspeptidase), nodules and tumors are quantified and evaluated. Initial studies are using TCDD and 17 α -ethinylestradiol (EE₂) as prototypical chemicals.

II. Develop and/or apply methodology for quantifying total receptor, occupied receptor, and unoccupied receptor in cytosol and nuclei of tissues as well as in tissues slices and fluids at different stages of tumor development.

III. Develop and/or apply biochemical and histochemical methods to evaluate changes in growth factor receptor and oncogene expression at various stages of tumor promotion.

IV. Develop methods to separate preneoplastic liver cells from normal parenchymal cells for the purpose of detecting early changes in gene expression. These methods will involve cell-sorting as well as histochemical approaches and will be useful in detecting early changes in gene expression in "preneoplastic" cells.

V. Determine at which stage tumor promoters are reversible following cessation of exposure using histological and biochemical parameters to evaluate reversibility.

VI. Attempt to evaluate the quantitative relationship between dose, receptor occupancy, DNA binding, protooncogene activation and tumor promotion. Our goals are to identify parameters using the rat two-stage model to determine similarities and differences between (1) various classes of promoters, (2) structural analogs of a given class of promoter, (3) promoters and initiators and (4) promotion of background initiated cells and chemically-initiated cells. These studies should allow us to more precisely quantify "biologically effective dose", elucidate the immediate biochemical consequences of this dose in a tumor promotion model and to more clearly define dose-response relationships.

VII. A number of parameters are to be quantified in order to accomplish our goals and these are: 1) Body and organ weights, 2) arylhydrocarbon hydroxylase activity (TCDD only), 3) concentration of TCDD or EE₂, 4) GGT positive foci (number and size), 5) specific receptors for EE₂ and TCDD, 6) EGF receptor binding and phosphorylation, 7) DNA synthesis, 8) oncogene expression, 9) histological foci, adenomas and hepatocellular carcinomas and 10) immune competence. The rationale for measurement of some of these parameters is described as follows:

Body and Organ Weights. Animal and selected organ weights are well established parameters that are useful for evaluating animal response to toxic agents. They are used as positive markers of exposure in our studies although they are not important mechanistic markers. For our tumor promotion studies, we are measuring body weight (known to be decreased by TCDD), liver weight (increased by TCDD), thymus weight (decreased by TCDD) and uterine weight (increased by estrogens).

Aryl Hydrocarbon Hydroxylase Activity (TCDD only). The most studied response elicited by TCDD is the induction of the hepatic microsomal enzyme, aryl hydrocarbon hydroxylase (AHH). Essential to the induction process is the existence of a cytosolic Ah receptor which binds TCDD and related halogenated aromatics. In fact, structure activity studies demonstrate that there is a good correlation between the rank order of binding to the cytosolic Ah receptor, the induction of AHH, and toxic responses such as lethality, thymic atrophy and teratogenicity. These studies have been important in establishing this relationship between structure, receptor binding and toxicity. Our goal is to determine whether a similar correlation can be demonstrated for promoting activity of TCDD and related compounds. In other words, to determine whether there is a relationship between receptor binding, induction of AHH and tumor promotion. It may turn out that AHH is a good marker of exposure but is unimportant to the tumor promotion process in our model system. If AHH induction is associated with tumor development, then specific P-450 isozymes will be quantified by "Western Blot Analysis" in collaboration with Dr. Philpot of the NIEHS.

Determination of TCDD or EE₂ Concentrations in Animals During Promotion. It is important to obtain information of the body burden of the promoting agents during the carcinogenic process. Because the liver is one of the primary storage sites for unmetabolized TCDD, we are analyzing TCDD concentrations in liver at specified intervals during promotion. For the estrogen promotion studies, EE₂ levels are measured in both serum and liver and serum values are compared to women who chronically use oral contraceptives.

Analysis of GGT-Positive Foci. Foci of altered liver parenchyma are detectable early during hepatocarcinogenesis. These foci are distinguished from surrounding normal liver tissue by a number of phenotypic markers that can be revealed by special techniques of which histochemical enzyme analysis has been particularly useful. The enzyme-histochemical techniques allow the detection of very small foci of altered cells. These histochemical markers are used to quantitate the number of altered foci. Enzyme altered foci are greatly increased in number, size and phenotypic heterogeneity by both TCDD and estrogens. Staining for gamma-glutamyltranspeptidase (GGT) is the single most useful method for quantitation, since over 90% of the foci and nodules are positive. Therefore, we are using GGT analysis to quantitate TCDD and estrogen promoted-preneoplastic lesions that develop during our initiation-promotion studies.

TCDD and Estrogen Receptors. The specificity of promoting agents for tissues may be related to their interaction with specific receptors in the target

tissues. Many responses to both TCDD and estrogens are mediated through the binding of specific cellular receptors. Therefore, the promoting action of these agents in hepatocellular carcinogenesis may require specific receptor binding. One of our primary goals is to determine whether there is a correlation between receptor binding, the induction of certain biochemical responses, and tumor promotion. This should establish whether interaction of these agents with a specific receptor pathway is important for their promoting action. If there is a direct relationship between receptor occupancy and tumor promoting ability, the threshold for these promoters can be determined; that is, the minimal amount of receptor occupancy required to promote tumor development. We are quantifying estrogen receptor concentrations and occupancy in cytosolic and nuclear fractions of both isolated parenchymal cells and whole liver during estrogen promotion. Likewise, liver TCDD receptor concentrations are measured throughout the TCDD promotion studies.

EGF Receptor Binding and Phosphorylation. There is general agreement that the basic defect in neoplastic cells is a loss or an alteration of the normal growth control mechanisms. Certain transformed cells have relaxed cell cycle controls and may traverse the cell cycle in the absence of exogenous growth factors. Recent data links certain oncogene encoded proteins to different compartments along the mitogenic pathway. Inappropriate expression of any of the control elements of the normal mitogenic pathway may result in autonomous growth. Some of these elements are growth factors, growth factor receptors, or intracellular signals which are involved in transducing growth factor signals to the nucleus resulting in DNA synthesis and cell divisions. We propose to investigate whether there is modification of growth factor receptor pathways during TCDD and EE₂ promotion that may possibly play an integral role in the promotion process. We are presently investigating whether there are alterations of the epidermal growth factor receptor (EGF-R) during the course of chemical hepatocarcinogenesis using the two-stage model. We are analyzing EGF receptor number, affinity, phosphorylation and mRNA levels. There are a number of reasons why we chose to investigate the EGF-R. 1) EGF-R has been associated with two oncogenes "ERB" and "NEU". 2) High mRNA levels and/or amplification of the EGF-R gene has been found in several different types of human tumors indicating that modification of the EGF-R plays same role in human tumor development. 3) EGF promotes skin tumors in mice initiated with 3-MC. 4) Total or partial loss of EGF receptors have been reported in chemically and spontaneously transformed liver cells. An excellent correlation between tumorigenicity and decreased EGF-R is found. 5) During 2AAF induced hepatocarcinogenesis, an early and sustained decreased in EGF-R is found. 6) High doses of TCDD produces a significant decrease in EGF-R binding and stimulated phosphorylation of the EGF-R. Some of the toxic manifestations of TCDD may be the result of action of TCDD on the EGF-R. 7) The liver is a responsive tissue to epidermal growth factor. There is some evidence that EGF or EGF-like factors are physiologic regulators of hepatocyte proliferation. EGF stimulates DNA synthesis in liver cells both in animals and in cell culture. In regenerating liver, EGF-binding is decreased indicating EGF or TGF factors may be causing down regulation. These studies are of interest because there appears to be a correlation between EGF, EGF-R and liver carcinogenesis. We are in a situation where we can determine whether modification of this receptor pathway occurs during liver tumor promotion.

Oncogene Expression. Transforming genes have been identified in a significant number of human cancers as well as in carcinogen-induced animal tumors and in vitro transformed cells. At present, more than 20 oncogenes have been identified by their association with RNA Tumor Viruses or by DNA-mediated gene transfer. The normal cellular counterpart called proto-oncogenes are highly conserved and evidence suggests they have crucial roles in normal growth and differentiation. The conversion of protooncogenes to transforming genes is called activation. Activation may be the result of increased dosage of a given protooncogene product (quantitative) or by mutations or rearrangement of the protooncogene (qualitative). Transformation of a normal cell into a tumorigenic cell may involve the activation and concerted expression of several protooncogenes. Carcinogen-induced animal tumor systems have long been considered appropriate models for human neoplasia. Cellular transforming genes have been identified in a variety of in vitro as well as in vivo animal model systems. In a number of cases, ras genes have acquired transforming properties by a single point mutation within their coding sequences. The activation of ras oncogenes in these systems is very reproducible. Therefore, animal model systems are suitable to study the role of oncogene activation during multistep carcinogenesis.

We are examining oncogene activation and expression throughout tumor development using different promoters. The NIH3T3 system is being used to detect activated oncogenes. Expression is being studied using specific cDNA probes and antibodies against specific oncogene encoded proteins. Modification of proto-oncogene mRNA levels may be the result of gene amplification, alteration of methylation patterns and/or of DNase I hypersensitive sites. These are being investigated to provide a better understanding of the mechanism by which the expression of these genes are altered during promotion.

Immunologic reagents have been considered to be useful in diagnostic histopathology especially in the detection of specific markers thought to be present on premalignant or malignant cells. This technique is particularly useful when only a small percentage of cells contain the antigen in question. We are utilizing antibodies specific to several different oncogene-encoded proteins to identify alterations in foci at very early stages of promotion that may be essential for further tumor progression. However, we are not limiting these studies only to oncogene encoded proteins but we plan to study a series of proteins whose expression has been associated with growth, differentiation and transformation of liver cells.

DNA Synthesis. There is evidence that liver promoting agents enhance the growth of cells in foci. Cells in foci are often found to have increased mitotic indices indicating that these lesions developed by the proliferation of altered hepatocytes. The acquisition of abnormal growth potential in response to promoters could involve modification of normal growth controls and may be a crucial event in promotion. We are measuring DNA synthesis and mitotic activity during our initiation-promotion studies. From such studies, we can determine if there is an association between DNA synthesis, growth factor receptor changes and oncogene expression.

MAJOR FINDINGS AND PROPOSED COURSE:

Our studies are still at the early stages of development such that the data presented here is still preliminary and many goals have not yet been accomplished. However, we have established the rat two-stage model for hepatocarcinogenesis and both TCDD and EE₂ are effective promoters in this system.

Estrogen Promotion: In our studies, ovariectomized Sprague-Dawley rats were administered a single dose of DEN (200 mg/kg, i.p.) in saline (s). Silastic capsules containing EE₂ were implanted subcutaneously to produce an estimated dose of 90 µg EE₂/kg/day and a serum concentration of 65±24 pg EE₂/ml. This concentration is equivalent to that of women who chronically use oral contraceptives. A progression of liver lesions (GGT positive foci, nodules, adenomas and hepatocellular carcinomas) was observed in initiated animals promoted with EE₂ for 40 weeks. Quantitative image analysis of GGT-positive foci indicates that the number of foci in initiated/promoted animals is not different than that of initiated controls at 10, 20 and 40 weeks of EE₂ treatment, but that the area of foci per area of tissue in initiated/promoted animals is much greater than that of initiated controls at 40 weeks; values (area of foci/area of tissue x 10³) are: DEN/EE₂, 24.0±14.0; DEN cholesterol control, 2.8±1.9. Virtually no foci were present in rats receiving only EE₂. This data suggests that estrogen promotion increases the growth potential of altered cells.

We have also examined the reversibility of hepatic GGT-positive foci upon cessation of EE₂ treatments in rats initiated with diethylnitrosamine. Promotion with EE₂ for 20 weeks and then withdrawal of treatment for 20 weeks does not yield significantly smaller area of foci/area of tissue than a continuous 40 week treatment. Thus, GGT-positive foci persist, and in fact increase in number and size, upon cessation of EE₂ treatments after 20 weeks suggesting that these lesions are not reversible at this stage of promotion. Hepatic ER concentrations and occupancy were quantified in both cytosolic and purified nuclear fractions of whole liver by an exchange assay and an enzyme immunoassay. After 10 and 20 weeks of EE₂, total nuclear ER values were not significantly different among treatment groups. However, receptor occupancy values in fmoles ER/mg DNA at 20 weeks were: S/C, 0.6±14.4; S/EE₂, 35.2±46.2; DEN/C, 46.8±26.7; and DEN/EE₂, 91.0±80.7. It appears that a single dose of DEN produces a change in the capacity of the nucleus to retain estrogens which may be related to the tumor promotion of EE₂ in the rat model. This may produce liver cell subpopulations which are particularly sensitive to exogenously administered estrogens and may be related to the tumor promoting activities of EE₂ in the rat two-stage model for hepatocarcinogenesis.

For this reason, the role of the hepatic estrogen receptor (ER) during the promotion phase has also been investigated in isolated liver cells. Hepatocytes were isolated by collagenase perfusion from adult ovariectomized Sprague-Dawley rats treated with a single initiating dose of DEN (200 mg/kg, i.p.) or saline (s) followed by chronic administration of EE₂ (20 weeks). ER levels were increased in the EE₂ and DEN treatment groups with DEN/EE₂ displaying the

greatest increase in both the cytosolic and nuclear subcellular fractions, therefore, an initiating dose of DEN enhances the concentration of ER attained by the parenchymal cells. In addition, ER receptor concentrations will be examined in the hepatic nonparenchymal cells, as well as enzymic activities of GGT and glucose-6-phosphatase. Enriched liver cell subpopulations which are particularly sensitive to the promotion process will be evaluated for biochemical changes such as receptor occupancy and oncogene expression which might be important to the mechanism of action of promoters.

TCDD Promotion. Ovariectomized adult Sprague-Dawley rats were administered an initiating dose of diethylnitrosamine (DEN, 200 mg/kg, i.p.) or saline, then promoted with doses of TCDD equivalent to 10, 30 and 100 ng/kg/day or corn oil (CO). After 22 weeks of TCDD promotion, dose dependent increases are observed in liver cytosolic TCDD receptor concentration (6.2 fmoles/mg to 13.8 fmoles/mg) and Arylhydrocarbonhydroxylase (AHH) activity (60 pmole/mg/min to 480 pmole/mg/min). Hepatic GGT positive foci are observed more frequently in initiated rats than in non-initiated rats and no foci were observed in control rats. Values (measured as #GGT foci per cm² tissue) were DEN/CO=3.0, DEN/TCDD=10.3, Saline/CO=0, Saline/TCDD=2.0. These changes may be associated with early stages of tumor progression in that there appears to be a good correlation between TCDD receptor concentration, the induction of AHH and GGT-positive foci. Further studies are evaluating TCDD receptor action in preneoplastic liver cells to determine if increased receptor concentrations in these cells is related to the proliferative actions of TCDD in the tumor promotion model.

Measurement of the EGF Receptor and its Phosphorylation. Our initial studies of TCDD effects on the EGF receptor were performed using male rats. We found that TCDD treatment (25 µg/kg) caused a significant induction of AHH activities and a significant reduction of EGF bindings, thus confirming earlier reports by Matsumura (1985). Plasma membranes isolated from ovariectomized females have significantly lower levels of EGF receptors (<50% decrease) than found in males, therefore, we have optimized the membrane isolation and assay conditions such that small changes in EGF-receptors may be detected following TCDD treatment. Preliminary results indicate that during EE₂ promotion, EGF receptor levels increase in a dose dependent manner. TCDD has differential effects on the EGF receptor; in DEN initiated livers there is an elevation of receptor levels; whereas, in control livers, TCDD treatment results in a decrease of EGF receptor. These studies are still ongoing and will indicate whether quantitative measurement of EGF receptor binding can be used as a sensitive means of detecting TCDD or estrogen effects at early as well as late stages of promotion.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Although the exact molecular mechanism of action of promoting agents has not yet been defined, alteration of gene expression by promoting agents appears to be essential to the promotion process. It has been proposed that promoters alter gene expression by a variety of mechanisms, including interaction with specific receptors or with cytoplasmic and nuclear components. Promoting agents elicit pleiotropic cellular responses of which only some are specific and crucial for

promoting activity. Because of this, we have proposed to investigate a number of carefully selected parameters and to correlate these with promoting activity.

2,3,7,8-tetachlorodibenzo-p-dioxin (TCDD) and related compounds such as some halogenated biphenyls and dibenzofurans are a highly toxic class of environmental contaminants as evidenced by numerous cases of accidental poisonings of human and animal populations and these compounds possess extreme toxic potency in laboratory animals including tumor promoting effects. In laboratory animals, histopathologic and biochemical effects are tissue and species specific. The spectrum of organ systems affected is different for different species. A considerable amount of effort has been expended on research projects which have attempted to characterize a common mechanism of action for the toxic halogenated aromatics. These studies have led to the discovery of a cytosolic binding protein or receptor for TCDD. Several lines of evidence suggest that this receptor is essential for the initiation of toxic sequelae that follow exposure to TCDD and related compounds.

The proposed model for the mechanism of action of TCDD and related compounds was derived from models for steroid-hormone receptors. According to the dioxin model, TCDD enters the cell and binds to its high affinity receptor. This receptor is selective for TCDD and related compounds; i.e., it does not bind steroid hormones or other compounds that do not produce the spectrum of biochemical and histopathological effects characteristic of TCDD exposure. The TCDD receptor has a finite capacity (fmol/mg cytosol protein) which leads to saturation at low concentrations of ligand allowing maximal responses at low doses. It is thought that the dioxin receptor complex binds to specific sites on chromatin thereby modulating gene expression producing induction and/or repression of synthesis of critical macromolecules.

This receptor model is attractive in its simplicity but unfortunately it does not provide a unifying hypothesis for all biochemical and toxic effects associated with exposure to TCDD. For example, there are huge species variations in susceptibility to hepatic enzyme induction, lethality and histopathologic effects in spite of the presence and similarity of properties of receptor in these species. In other words, the correlations that exist between cytosolic receptor concentrations and toxicity in inbred strains of mice are not present in other species. Moreover, species differences in metabolic and clearance rates are relatively small and cannot account for species variations in toxicity. These findings suggest that a primary factor in determining toxicity might be tissue and species specific factors that control the actions of receptor in target tissues. Identification of the tissue specific factors, which are important to tumor promotion, is an essential component of our studies. For the reasons detailed in the Objectives and Methods section, we are investigating a number of biochemical parameters that seem essential to the tumor promoting process. Oncogene expression and growth factor actions are particularly relevant. These data coupled with exposure data should eventually allow us to estimate the biologically-effective dose of TCDD and related compounds required to produce a specified risk to tumor promotion.

The mechanism of action of estrogenically-active compounds also operates through a receptor system. We feel that interactions of this receptor system, like the dioxin receptor, is an essential step in the tumor promoting activities of estrogens. Accordingly, characterization of the quantitative relationships between exposure, receptor occupancy, gene expression and tumors should enhance our ability to devise rational risk assessment approaches. Of particular importance will be our ability to address the questions of 1) reversibility of preneoplastic lesions induced by classic tumor promoters and b) the dose-response relationships for these compounds using "biologically-effective dose" as well as the biochemical consequences of this dose as the indicators of exposure.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 ES 46005-01 LBRA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oncogene Activation and Expression in Rodent Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Steve Reynolds	Staff Fellow	LBRA	NIEHS
	Marshall Anderson	Res. Mathematician	LBRA	NIEHS
Others:	J. Stowers	Chemist	LBRA	NIEHS
	S. Belinsky	NIH Postdoctoral	LBRA	NIEHS
	J. Angerman-Stewart	Biologist	LBRA	NIEHS
	R. Patterson	Microbiologist	LBRA	NIEHS

COOPERATING UNITS (if any)

Dr. Robert Maronpot, National Toxicology Program, NIEHS
Dr. Stuart Aaronson, National Cancer Institute

LAB/BRANCH

Laboratory of Biochemical Risk Analysis

SECTION

Molecular Toxicology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

3.5

2.5

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Recent evidence from two independent lines of investigation has merged to suggest that neoplasia results from the abnormal activation of a relatively small number of cellular genes. Certain retroviruses contain transduced cellular genes which infer transforming properties to the retrovirus. The retroviruses containing these cellular sequences in their genome can induce tumors in animals or transform cells in vitro. Subsequent studies have established that proto-oncogenes can also be activated as oncogenes in naturally occurring tumor cells by mechanisms completely independent of retroviral involvement. These genetic alterations range from point mutations to gross DNA rearrangements such as translocation and gene amplification. We have recently initiated studies to investigate oncogene activation and expression in spontaneous and chemical-induced tumors in rodents. Comparison of types and extent of oncogene activation in spontaneous versus chemically-induced tumors may aid in risk assessment of chemicals based on bioassay data obtained from various animal model systems. In addition, animal model systems must be developed to study the sequence of oncogene activation and expression.

PROJECT DESCRIPTION

OBJECTIVES AND METHODS EMPLOYED:

1. To examine oncogene activation in spontaneously arising tumors in animals and to compare with oncogene activation in chemically-induced tumors. Comparison of types and extent of oncogene activation in spontaneous versus chemically-induced tumors may aid in risk assessment of chemicals based on bioassay data. Also, the suitability of various animal model systems for bioassay studies may be clarified.
2. To examine tissue and chemical selectivity for oncogene activation in chemically-induced tumors.
3. To investigate the kinetics of oncogene activation. To establish the earliest time after carcinogen administration at which a transforming gene can be detected and/or abnormal expression of various oncogenes.

The experimental approaches used to investigate these objectives are as follows:

1. Detection of transforming gene in tumor tissue DNA by transfection assay: We will analyze for transforming genes in the tumor tissue DNA by the standard NIH/3T3 transfection assay.
2. Identification of activated oncogene by southern blotting analysis: To identify the transforming genes we will isolate DNA from transfectants and subject the DNA to southern blotting analysis using known oncogenes as DNA probes. If the transforming gene in tumor tissue is not identified as a known oncogene, we will have to molecularly clone the transforming gene according to established techniques.
3. Analysis of protein product of activated ras oncogenes: Recent reports have shown that ras genes which have undergone mutational activation frequently produce P21 proteins with altered electrophoretic mobilities. Characteristic alterations of either slower or faster mobility appear to accompany many of the activating lesions at positions 12 and 61, respectively. Therefore, first or second cycle DNA transfectants will be analyzed for the presence of ras proteins with altered electrophoretic mobility.
4. Analysis of transforming gene for mutations: The oncogene from transfectant DNA and from normal tissue DNA will be cloned and sequenced to determine if the oncogene was activated by a point mutation. Oligonucleotide probes centered around the codon containing the mutation will be used to analyze the tumor DNA.
5. Analysis of oncogene expression in normal, preneoplastic, and neoplasia tissues: Total cellular and poly A (+) RNA from normal, preneoplastic, or tumor tissue will be isolated. Northern blot analysis will be performed to quantitate expression of various oncogenes in these tissues at specified times after chemical treatment.

MAJOR FINDINGS AND PROPOSED COURSE:

1. Species and strain-specific occurring tumors have been observed in rodents maintained under normal laboratory conditions. In view of the high frequencies of oncogene activation shown in rodent tumors induced by known chemical carcinogens, we have investigated oncogene activation in spontaneous tumors of the B6C3F1 mouse and Fischer 344/N rat by DNA transfection techniques. A marked difference in the presence of activated oncogenes in spontaneous rat tumors versus spontaneous mouse liver tumors was observed in this study. All rat tumors tested failed to yield activated oncogenes (0/29), whereas 30% (3/10) of mouse hepatocellular adenomas and 77% (10/13) of hepatocellular carcinomas scored positive by DNA transfection. These dominant transforming genes were identified as an activated H-ras gene in all the adenoma transfectants and 8 of the 10 carcinoma transfectants. The two remaining hepatocellular carcinomas contained transforming genes which were not members of the known ras gene family. The B6C3F1 mouse liver system might provide a very sensitive assay for assessing not only the potential of a chemical to activate a cellular proto-oncogene, but also to detect various classes of proto-oncogenes which are susceptible to mutational activation. Comparisons of the pattern of oncogene activation between spontaneous and chemically-induced mouse liver tumors should aid in the interpretation of bioassay data.
2. We have examined tetranitromethane-induced rat lung tumors for the presence of transforming genes. Fourteen out of nineteen tumor samples induced morphological transformation of NIH/3T3 fibroblasts in this assay while normal rat lung tissue did not. Southern blot analysis of secondary transfectants showed that the transforming properties of the rat tumor DNA were due to the transfer of an activated cellular homolog of the K-ras oncogene. Radioimmunoprecipitation and SDS-polyacrylamide gel electrophoresis showed that the ras proteins from these secondary transfectants did not have altered mobility as has been reported previously for some activated ras proteins. Because a structural mutation could not be shown in the transfected K-ras gene product, dot blots were performed on the original tumor DNA and showed no amplification of the normal K-ras gene. This study shows reproducible activation of the K-ras oncogene by the chemical, TNM, in three different tumor types (squamous cell carcinoma, adenocarcinoma, and mixed tumor type). Further studies are currently being pursued to elucidate the nature of the activating lesion in the transfected K-ras oncogenes. These results in rat lung tumors are similar to published results in human lung tumors and cell lines derived from lung tumors.

Plans for the subsequent year are as follows:

1. We will characterize the activated oncogenes in furfural- and furan-induced mouse liver tumors. The pattern of oncogenes present in these chemically-induced tumors in the B6C3F1 mouse will be compared to those already identified in the spontaneously occurring liver tumors. This will be the first attempt to compare spontaneous rodent tumors with chemically-induced tumors.

2. We will attempt to identify the mutation in the activated K-ras gene present in pulmonary tumors from tetranitromethane treated animals. If a point mutation is found, then the original tumor DNA will be probed with an oligonucleotide probe in order to determine if the same mutation is present in all of the tumors.
3. Transforming genes have been observed in mouse liver tumors which are not members of the known ras gene family. We will attempt to characterize these unknown transforming genes.
4. We will examine a variety of spontaneously occurring tumors in the B6C3F1 mouse for the presence of transforming genes. This is a continuation of a previous study which identified activated oncogenes in spontaneously occurring mouse liver tumors.
5. We have DEN-induced hepatocellular carcinomas (adenomas) in mice and rats. The rat liver tumors were induced by either continuous exposure to DEN in drinking water or by an initiator-promotor protocol with various promoters. The mouse liver tumors were induced by a single dose of DEN to 12 day old animals. We are presently comparing the various types of DEN-induced tumors for oncogene activation and expression.
6. We have a variety of mammary tumors in rats, both spontaneous and chemically-induced. Chemicals include DEN, MNU, DMBA, BP and various benzidine derivatives. We will examine these tumors for oncogene activation and expression.
7. Several benzidine derivatives have been tested in the bioassay program. These compounds produced numerous tumors in rats. We are presently examining these tumors for oncogene activation and expression.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Recent evidence from two independent lines of investigation has merged to suggest that neoplasia results from the abnormal activation of a relatively small number of cellular genes. Certain retroviruses contain transduced cellular genes which infer transforming properties to the retrovirus. The retroviruses containing these cellular sequences in their genome can induce tumors in animals or transform cells in vitro. Also, DNA from human tumor tissue and chemically-induced animal tumor tissue contain dominant-acting sequences which, when incorporated by gene transfer techniques into contact inhibited NIH/3T3 cells, will induce neoplastic transformation. The transforming gene detected by the gene transfer procedure have been shown, in most cases, to be analogs to the transduced cellular sequence of a retrovirus. These transforming genes are called oncogenes and each oncogene is homologous to a counterpart DNA sequence present in the normal genome (proto-oncogene). The proto-oncogenes do not have transforming activity themselves. Proto-oncogenes have been found in species as divergent as *Drosophila* and man. Approximately two dozen proto-oncogenes have been discovered to date. The highly conserved nature of these proto-oncogenes

suggests that the protein products they encode have crucial roles in normal cellular function and differentiation. Recent work suggests several mechanisms for the conversion of proto-oncogenes to active oncogenes, including gene mutation, gene amplification, chromosomal rearrangements, and promoter insertion. For example, the sequences responsible for the transforming activity of several human tumors and animal tumors have been identified as mutated forms of the proto-oncogenes of the ras family of retroviral oncogenes. In human lung, colon, and bladder carcinomas, either the Ha-ras or K-ras proto-oncogene was converted into a potent oncogene by a single point mutation. Rat mammary carcinomas induced by MNU contained an Ha-ras oncogene which differed from the proto-oncogene by a single point mutation. As another example, chromosomal translocation of the myc oncogene appears to be involved in certain types of lymphomas. The number of proto-oncogenes that must be activated in order to convert a normal cell into one that is tumorigenic is unknown at present.

The possibility that the transformation of a normal cell into a tumorigenic cell involves the activation and concerted expression of several proto-oncogenes may provide new insights into long-standing problems in chemical carcinogenesis. Firstly, the tissue (cell) selectivity of certain carcinogens may be reflected in their inability to activate all of the required oncogenes in the resistant tissues. Alternatively, the set of oncogenes activated by the carcinogen may be the same in both susceptible and resistant tissues but the concerted expression leads to neoplasia only in certain tissues. Secondly, the classification of chemicals as initiators, promoters, complete carcinogens, etc., may become clearer as we better understand the sequential requirements for activation of oncogenes in the various animal model systems. Thirdly, low-dose and species-to-species extrapolation of bioassay data may become more reliable from examination of oncogene activation and expression in animal model systems for carcinogenesis.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 46006-01 LBRA																														
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PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">Thomas Wong</td> <td style="width: 20%;">Staff Fellow</td> <td style="width: 15%;">LBRA</td> <td style="width: 15%;">NIEHS</td> </tr> <tr> <td></td> <td>George W. Lucier</td> <td>Chief</td> <td>LBRA</td> <td>NIEHS</td> </tr> </table> <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">Others:</td> <td style="width: 40%;">G. Sunahara</td> <td style="width: 20%;">Visiting Fellow</td> <td style="width: 15%;">LBRA</td> <td style="width: 15%;">NIEHS</td> </tr> <tr> <td></td> <td>K. Nelson</td> <td>Staff Fellow</td> <td>LBRA</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>T. Sloop</td> <td>Biologist</td> <td>LBRA</td> <td>NIEHS</td> </tr> <tr> <td></td> <td>M. Anderson</td> <td>Res. Mathematician</td> <td>LBRA</td> <td>NIEHS</td> </tr> </table>			PI:	Thomas Wong	Staff Fellow	LBRA	NIEHS		George W. Lucier	Chief	LBRA	NIEHS	Others:	G. Sunahara	Visiting Fellow	LBRA	NIEHS		K. Nelson	Staff Fellow	LBRA	NIEHS		T. Sloop	Biologist	LBRA	NIEHS		M. Anderson	Res. Mathematician	LBRA	NIEHS
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COOPERATING UNITS (if any) Epidemiology Branch, BRAP Laboratory of Pharmacology, IRP Wright State University (M. Taylor)																																
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The objectives of this project are to evaluate the congener-dependency of some of the biochemical effects observed with human exposure to halogenated aromatics and the role of receptor(s) in mediating the biologic and toxic effects in placental tissue. This information will be critical in determining mechanisms of individual differences in responsiveness to the chlorinated chemicals. The metabolic activation and the role of receptor(s) in mediating the biochemical effects in human placentas are the central focus of this project. Study subjects were pregnant women identified from a registry of individuals accidentally exposed to PCBs, PCDFs, and PCQs; control subjects were age-matched to within three years of exposed subjects. Placental homogenates and microsomes from exposed subjects showed marked elevation of cytochrome P-450 monooxygenase activities when compared with samples from controls. "Western blots" of placental microsomes were found to contain a protein which cross-reacted with antibody raised to rabbit cytochrome form 6, an isozyme induced by polycyclic aromatic hydrocarbons. The elevation of P-450 dependent monooxygenase showed that the biologic effects resulting from this accidental exposure (in Taiwan in 1979) can persist for at least 5 years. No relationship between the biochemical markers studied and blood PCB levels and/or clinical symptoms of exposed subjects is apparent. A consistent finding is that birth weights of offspring were lower for exposed subjects than control subjects. Work is in progress to explore the role of receptors in mediating this metabolic alteration in placentas as well as in determining the persistent PCB and PCDF congeners contributing to these biologic effects. </p>																																

PROJECT DESCRIPTION

OBJECTIVES AND METHODS EMPLOYED: The general objective is to characterize some of the biochemical abnormalities associated with human exposure to polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and polychlorinated quaterphenyls (PCQs) in a 1979 accidental food poisoning episode in Taiwan, determine the congener dependency of some of these effects, and propose likely mechanism(s) mediating the human biologic responses to these halogenated aromatics. The specific objective are as follows:

- I. Evaluate the use of select cytochrome P-450-dependent enzyme activities in human placentas from exposed and control subjects as "biochemical markers" of exposure to halogenated aromatics and the utility of placental enzyme markers for the purpose of cross-species comparison.
- II. Correlate the quantification of placental enzyme markers with clinical findings of exposed subjects and their newborn(s) and the immunochemical measurement of human cytochrome P-450 isozymes selectively altered in placentas of human subjects (with antisera to several rabbit cytochrome P-450 isozymes). In experimental animal systems, PCBs and structurally-related compounds are known to alter concentrations of specific cytochrome P-450 isozymes. The cytochrome P-450 monooxygenase system is extraordinarily complex so by measuring the amount of induced protein rather than a P-450 dependent enzyme such as aryl hydrocarbon hydroxylase (AHH) we should enhance our efforts to evaluate dose-response relationships when using biochemical markers in placenta as indicators of exposure.
- III. Develop methodology for measurement of receptor(s) in human placenta for purpose of determining the role of receptor(s) in mediating the clinical and metabolic abnormalities associated with halogenated aromatic hydrocarbon exposure and whether receptor level(s) serves as determinant of inter-individual variation in the biologic response. Specific receptors to be measured include the Ah and epidermal growth factor (EGF) receptors. These receptors are proposed to act as mediators at the transcriptional levels for many biochemical responses to several classes of chemical compounds. The Ah receptor(s) is thought to mediate induction of aryl hydrocarbon hydroxylase (AHH) by halogenated aromatics through a mechanism involving increased synthesis of a specific form of cytochrome P-450. This receptor has been identified and characterized in hepatic cytosol using the model polychlorinated dibenzo-p-dioxin (PCDD), 2,3,7,8-TCDD. This model dioxin is also shown to reduce liver EGF receptor binding in animal models. The human placenta is an extremely rich source of EGF receptor; approximately 50 times higher concentrations are found in placenta compared to liver. Accordingly, decreased EGF finding in placentas of exposed subjects may provide a sensitive marker for exposure to toxic halogenated aromatics.

For comparison, TCDD and EGF receptor levels in placentas of women who smoked during pregnancy will be examined in a collaborative study (see project Z01 ES 43008-05 EB).

- IV. Perform chemical analyses of PCBs, PCDFs, and PCQs in blood and placental tissue samples of exposed and control subjects. The reason for this objective is that most researchers feel that the causative agent in the Yusho and Taiwan poisoning incidents was the PCDFs which were contaminants of the PCBs. These compounds are structurally similar to TCDD and they bind extremely well to the TCDD receptor. Therefore, we are developing methods for identification and quantification of persistent PCB and PCDF congeners. The goal is to determine the relationship among the blood and tissue levels of persistent PCDF and PCB congeners, the enzyme activities, the receptor levels, and the clinical findings of exposed subjects compared with control subjects.

MAJOR FINDINGS AND PROPOSED COURSE: Even four to five years after the food poisoning episode, placental homogenates of several Yucheng ("oil disease") subjects had dramatically elevated levels of AHH and 7-ethoxycoumarin o-deethylation (7ECD) activities compared with samples from nonexposed subjects; induction was approximately 100-fold. Since the AHH assay measures chiefly the formation of benzo(a)pyrene (BaP)-phenols, BaP metabolism *in vitro* was studied further by quantifying specific metabolites by HPLC. Little or no BaP metabolites were generated in samples from control subjects. Placental homogenates from Yucheng subjects generated larger amounts of 3-OH-BaP, BaP-quinones, and BaP-7,8-diol. BaP-4,5 diol and BaP-9,10-diol were also produced, but in lesser amounts. These results reveal that the metabolic effect of these chlorinated chemicals were highly persistent. For comparison, the HPLC profile of BaP metabolites generated by placental homogenates of smokers resembled that of samples from Yucheng subjects (see Z01 ES 43008-05 EB).

Clinical findings for the several Yucheng and control subjects and their newborn(s) were abstracted from the medical charts. Additionally, we abstracted exposure data and clinical information on Yucheng subjects from the local PCB registry records. The exposed women in the study had mild to moderate signs and symptoms typical of clinical effects of exposure. One consistent finding was that birthweights of offspring were lower in Yucheng subjects than in nonexposed individuals.

In order to better evaluate dose-response relationships for PCB-mediated induction of placental enzymes, we treated pregnant Sprague-Dawley rats with several concentrations of Aroclor 1254 ranging from 0.5 to 500 mg/kg body weight and investigated the levels of AHH and 7ECD activities in maternal livers, placentas, and fetal livers. This study indicated that relatively low doses of PCBs were needed to increase levels of cytochrome P-450-dependent monooxygenase activities. Metabolic activity in maternal hepatic and placental tissue homogenates was induced by Aroclor 1254 dose of 15 mg/kg body weight. Placenta appeared as sensitive as maternal liver to enzyme inductive effects, although the basal level of enzyme activities in placenta were 2-3 orders of magnitude lower than liver. If this concentration can be extrapolated to humans weighing 50-60 kg, it would be equivalent to about 750-900 mg PCBs, an amount similar to the total dose which exposed subjects received over 8-9 months from the PCB-contaminated rice oils in Taiwan. In the rat study, transplacental induction of fetal hepatic enzymes required about an order of magnitude higher PCB dosage. A placental barrier to PCB transfer has been hypothesized to be one

explanation for the higher dose requirement for transplacental induction of fetal livers compared with maternal livers. In the Taiwan episode, enzyme induction in placental specimens of Yucheng subjects may have been due to PCBs, PCDFs, or PCQs or to a combination with these chemicals in the contaminated oil; the proportional contribution of each of the chemical classes is unknown. Enzyme induction was probably influenced by retention, mobilization, and inducing potency of specific isomers of these compounds.

Following these studies, we have used antibodies to rabbit P-450 isozymes to detect protein in human placental microsomes by "western blotting", a technique that couples electrophoretic transfer of protein bands to nitrocellulose paper with immunochemical detection. In this study, we report the induction of placental microsomal protein from Yucheng subjects immunochemically related to rabbit isozyme 6. Concentrations of this isozyme were elevated 150-fold in PCB-exposed placentas. This finding is supported by immunochemical and enzymatic evidence. No band of cross-reactivity and low AHH and 7-ethoxyresorufin o-deethylation (7ERFD) activities were found in microsomal samples from control subjects. Among Yucheng subjects, a significant correlation was found between the immunoreactivity of the cross-reactive protein and AHH and 7ERFD activities. The presence of other P-450 isozymes in the crude microsomal preparations from Yucheng and control subjects cannot be ruled out, although cross-reactivity with antibodies to rabbit isozymes 2 and 5 was not detected under our experimental conditions. The same isozyme 6-related protein was detected in placental microsomes from smokers but not in samples from nonsmokers although increases were less than those observed in PCB-exposed individuals. However, none of the Yucheng subjects was reported to have been a smoker during pregnancy. The presence of this isozyme 6-related protein in placentas from individuals exposed to used PCBs or from smokers and its absence in the placentas of respective nonexposed subjects indicated that the same P-450 isozyme(s) is induced by either chemical mixtures. However, there appeared to be no apparent correlation of the placental microsomal levels of rabbit isozyme 6-like protein with either blood PCB levels or the severity of clinical symptoms. These data suggest that either (a) P-450 induction has nothing to do with toxicity or (b) PCDFs or other compounds in the rice oil cause the biochemical and toxic effects, not the PCBs.

Studies on the determination of Ah and EGF receptors in human placentas are in preliminary stages. We have tested several published Ah receptor assay, including the dextran-coated charcoal assay, the hydroxyapatite assay, the protamine sulfate assay, and the sucrose density gradient centrifugation procedure. These methods all indicated that the concentrations of cytosolic Ah receptor(s) were uniformly low in human placentas from exposed and control subjects. We are now designing a protocol which will modify several of these methods and/or use a combination of assays to give a consistent measurement of the specific ligand binding to the Ah receptor(s). The availability of polyclonal receptor antibodies in the near future would enable the detection of nuclear receptor complexes not now possible with the tracer-based methods and would simplify the measurement of receptor levels, especially in clinical studies. For measurement of EGF ++ receptors in placental membrane fractions, we have found that inclusion of mg in the isolation buffer reduced the high affinity binding site(s) and have eliminated the bivalent action in our

membrane purification procedure. In preliminary experiments with placental membrane fraction from nonsmokers, the K_D for the high affinity binding site(s) was calculated to be 0.1 - 1 nM and for the low affinity binding site(s) was 1 - 10 nM. The membrane isolation procedure and the EGF receptor assay are now being refined and validated before drafting the final protocol to be used for measurement of EGF receptors in human placental samples.

PCBs, PCQs, and PCDFs are mixtures of various congeners. The toxicities and biologic behavior are congener-dependent in model animal studies. In this project, we are collaborating with Dr. Mike Taylor of Wright State University to evaluate methods to clean up biological matrices such as blood and placental tissues for PCB, PCDF, and PCQ analyses as well as procedures to follow for identification and quantification of specific PCB and PCDF congeners (PCQ congeners will not be done due to lack of standards). In these studies, we focus on the highly persistent congeners because our research is being conducted five years after the exposure had occurred. PCB and PCDF congeners of interest are those which are approximate isostereomers of 2,3,7,8-TCDD (with chlorine substituents in the lateral positions). Of particular interest are two pentachlorodibenzofurans which have estimated half-lives of 50 years and bind well to the TCDD receptor. The identification and quantification of these PCB and PCDF structurally related to TCDD will be correlated with other biochemical parameters and will contribute to better understanding of the biologically-effective doses of these chlorinated compounds needed to produce and maintain some of the chronic human biologic responses (e.g., P-450 enzyme elevation and clinical symptoms).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: PCBs are chlorinated-substituted biphenyls which had wide industrial applications as plasticizers, lubricants, heat-exchangers, and insulators. Although the commercial use of PCBs has been restricted in the U.S. since the early 1970s, these chemicals, which are highly resistant to degradation, continue to be dispersed in the environment and have been found to accumulate in the human body through the food chain. Much of what is known about human toxicity associated with exposure to PCBs and structurally related compounds have come from accidental occupational exposures and food poisoning episodes. The two food poisoning episodes of importance occurred in 1968 in Japan and in 1979 in Taiwan. In both episodes, subjects were exposed through consumption of contaminated rice oils. Patients with the "oil disease" (Yusho in Japanese and Yucheng in Chinese) suffered from various chronic symptoms such as acniform eruption, hypersecretion of the Meibomian glands, pigmentation of face, eyelids, gingiva, and nails. The rice oils consumed by patients were found to contain not only PCBs, but also PCDFs and PCQs, the latter two classes were produced by heating of PCBs at high temperatures. The unfortunate poisoning episode in Taiwan provides a unique opportunity to study a human population with high exposure to halogenated aromatics. A better characterization of the halogenated aromatic compounds involved in this exposure episode as well as some of the metabolic effects from such exposure would add to the understanding of how these chlorinated compounds contribute to the observed human toxicities. The use of human placentas are justified because the tissues, discarded after birth, is accessible, and therefore provides a source of tissue to investigate biochemical markers of exposure to toxic halogenated aromatics.

Halogenated aromatics such as PCBs, PCDFs, and PCDDs shared a number of common physiochemical, biological and toxic properties. These classes of compounds are a mixture of congeners. The toxic and biologic potencies of individual PCBs, PCDFs, and PCDDs are structure dependent with the most active compounds similar in structure to TCDD. One of the best studied biologic effects of these compounds similar in structure to TCDD is the induction of cytochrome P-448-dependent monooxygenases such as AHH. While a few studies have shown that AHH activity can be dissociated from the toxic responses, the induction potency of individual congeners of several PCBs, PCDFs, and PCDDs correlated well with their toxic potency. The initial step in enzyme induction appears to involve specific binding of the inducer(s) to a receptor protein. The receptor-ligand complex may bind the specific regulatory region(s) of DNA, increase production of specific RNAs coding for enzymes regulated by the Ah complex. The receptor has high affinity for TCDD, some PCDF and PCB congeners and for some polycyclic aromatic hydrocarbons. There appeared to be a good correlation between potencies of individual PCDD, PCDF, and PCB congeners to induce AHH activities and their affinities for receptor binding. The most active PCB and PCDF compounds are approximate isostereomers of 2,3,7,8-TCDD. Studies on structure-activity relationship of various classes of halogenated aromatic compounds, indicate that the basic requirement for ligand binding to the Ah receptors is a molecular structure which has a planar rectangle with halogen atoms in the four corners. The finding of persistent PCB and PCDF congeners in blood and tissue of Yucheng subjects which fit these requirements would aid in our understanding of the mechanisms mediating the pleiotrophic responses associated with halogenated aromatic exposure and be useful in evaluating the prognosis of patients with "oil disease"

PUBLICATIONS

Wong, T.K., Domin, B.A., Bent, P.E., Blanton, T.E., Anderson, M.W., and Philpot, R.M.: Correlation of placental microsomal activities with protein detected by antibodies to rabbit cytochrome P-450, isozyme 6 in preparations from humans exposed to polychlorinated biphenyls. Cancer Res. (submitted)

Wong, T.K., Blanton, T.E., Hunnicutt, C.K., and Everson, R.B.: Dose requirements, assay procedures, and tissue specificity for PCB induction of P-450 dependent monooxygenase activity in the rat: Implications for design of studies measuring in vivo induction of human placental monooxygenases. J. Appl. Toxicol. (in press)

Wong, T.K., Everson, R.B., and Hsu, S.-T.: Potent induction of human placental mono-oxygenase activity by previous dietary exposure to polychlorinated biphenyls and their thermal degradation products. The Lancet 1: 721-727, 1985.

Wong, T.K., Blanton, T.E., Hunnicutt, C.K., and Everson, R.B.: Quantitation of aryl hydrocarbon hydroxylase and 7-ethoxycoumarin o-deethylase activity in human placenta: Development of a protocol suitable for studying effects of environmental exposures on human metabolism. Placenta (in press).

Wong, T.K., Hunnicutt, C.K., Blanton, T.E., St. Clare, K., Cefalo, R.C., and Everson, R.B.: A modified protocol for measuring alteration in human placental aryl hydrocarbon hydroxylase activity associated with environmental chemical exposure. In Hayes, A.W., Schnell, R.C., and Miya, T.S. (Eds.): Developments in Science and Practice of Toxicology. North Holland, Elsevier Press, 1984, pp. 395.

STATISTICS AND BIOMATHEMATICS BRANCH

STATISTICS AND BIOMATHEMATICS BRANCH Summary Statement

The statistical and mathematical research conducted within the Statistics and Biomathematics Branch (SBB) can be grouped into the general categories of statistical applications, methodology development, and modeling. Specific research areas of continuing interest include statistical studies in carcinogenesis and genetic toxicology, mathematical modeling of molecular phenomena, risk assessment methodology development, and the generation of biostatistical procedures applicable to epidemiology and toxicology. Individual research projects are often collaborative in nature involving scientists from the Institute's Intramural Research and Toxicology Research and Testing Programs as well as individuals within BRAP's Epidemiology Branch and Laboratory of Biochemical Risk Analysis. Some of these individual projects are mentioned below together with a brief description of the underlying rationale that led to their initiation.

In the absence of epidemiological data, laboratory animal carcinogenicity studies provide the primary means of assessing a chemical's carcinogenic potential. SBB research in this area is concerned with improvements in the design, analysis, and interpretation of laboratory animal carcinogenicity experiments to enhance the value of these studies for assessing human health risk. The National Toxicology Program has generated a large database of laboratory animal carcinogenicity data that has been utilized in addressing important methodological issues such as assessing the impact of using maximum tolerated doses on the detection of carcinogenic potential, evaluating and controlling false positive rates in carcinogenicity testing, and modeling tumor onset distributions to further understanding of the development and progression of these lesions. Other issues under consideration include the assessing of synergism in carcinogenicity testing and the developing of improved techniques for analyzing non-fatal tumors.

Mutagenesis research and testing conducted by the NIEHS yields large amounts of data useful in the study of chemical mutagens, and the identification of appropriate, objective methods of analysis is acknowledged by most researchers in mutagenesis to be a clear and pressing need. Examples of SBB statistical methodology research that have been directed at this need include the development of methods for the analysis of in vitro cytogenetics data generated with chinese hamster ovary cells, and a critical reevaluation of the existing literature on sex and race effects on sister chromatid exchanges observed in human peripheral lymphocytes of control subjects. Attention has also been paid to the assessment of interlaboratory variability and interassay concordance, issues that impact on the credibility and usefulness of short-term tests for mutagens and carcinogens. The need to include in vivo short-term tests in the predictivity of carcinogenesis has been increasingly clear, and work is underway to evaluate existing methods of analysis and to develop new and improved ones where possible.

Within the SBB, research is also conducted on the mathematical modeling of biological processes at the molecular level. The methodology is now available to detect genetic variation at the DNA level, and consequently the implications of high observed levels of genetic variation for the future health of an individual can begin to be studied. The development of statistical methods for analyzing DNA sequence data and/or restriction map data is an important part of this endeavor. Reliable estimates of such quantities as mutation and recombina-

tion rates are important both to further understanding of molecular evolution in general and also for practical reasons such as the calculation of diagnostic rates in prenatal diagnoses of genetic diseases using genetic markers.

Risk assessment methodology development, which involves both laboratory-based and epidemiologic data, is primarily concerned with quantifying the potential risk of adverse health effects in humans as a result of exposure to various occupational and environmental agents. One of the major goals of this research effort is the reduction of some of the uncertainty in the risk assessment process through the development of more scientifically-based, biologically supportable methodology for risk estimation. Among the topics that are currently under study are the quantification of carcinogenic potency and relative potency, the evaluation of the impact of litter effects on teratogenic dose-response modeling, and the improvement of low-dose cancer risk estimation by the application of molecular epidemiologic techniques.

Statistical research in epidemiology has been carried out both to broaden the understanding of the uses and limitations of currently employed study designs and related analyses, and to develop new statistical methodology applicable to the field in general and to the epidemiologic interests of the Institute in particular. Specific issues that are currently under investigation include the development of models useful in the assessment of fertility and the evaluation of statistical methods that are presently employed in the analysis of spontaneous abortions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 40004-08 SBB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Methods in Epidemiology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Beth C. Gladen Statistician SBB NIEHS

Others: Clarice Weinberg Mathematical Statistician SBB NIEHS

COOPERATING UNITS (if any)

Epidemiology Branch, NIEHS
Cardio-thoracic Institute, University of London

LAB/BRANCH

Statistics and Biomathematics Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The purpose of this project is to conduct research on statistical methodology problems which arise in relationship to the Institute's activities in the field of epidemiology. Epidemiologic data, because of its observational nature, is subject to biases and other analytic difficulties not seen in experimental data. In addition, such data can be quite complex, with many complicating factors to account for. The objective of this project is both to broaden understanding of the uses and limitations of currently employed study designs and corresponding analyses, and to develop and evaluate new techniques for statistical analyses of epidemiological studies. Current research on this project involves (1) the development of methods for the assessment of fertility through the use of time-to-pregnancy data (i.e., number of menstrual cycles required to achieve pregnancy) (2) an assessment of some of the techniques currently in use for the analysis of spontaneous abortions to determine whether these techniques adequately handle the various sorts of biases which are involved in this type of data (3) the development of techniques for assessing the relationship between two processes (in this case, heart rate and respiration) and for using this relationship (or disturbances thereof) to study conditions such as sudden infant death syndrome.

PROJECT DESCRIPTION

METHODS EMPLOYED: In addressing the development or evaluation of methods for handling varied types of epidemiologic data, a variety of techniques will be required. Models for various types of data encountered in epidemiologic studies are developed through a combination of assessment of the important biological features of the data and attention to the mathematical tractability of the proposed model. Some of the results we achieve can be obtained by straightforward mathematical analysis of a proposed model or statistical test. The adequacy or inadequacy of proposed statistical models can be tested by a comparison of theoretically obtained results to actual results in existing data sets. In some cases, the complexity of an existing or proposed statistical procedure is such that simulation techniques are required in order to determine the properties of the procedure. All of these techniques have been required on occasion in the course of this research.

MAJOR FINDINGS AND PROPOSED COURSE: 1) Methods for assessing fertility are currently not well studied. One way to look at fecundity (i.e., the biological capacity to conceive children) is to study the number of menstrual cycles required to achieve pregnancy. Statistical models were developed for data of this sort. It was assumed that fecundity varied from couple to couple according to a beta distribution, so that the time required to achieve pregnancy had a beta-geometric distribution. Methods for estimating the parameters of such a model were developed. Methods for estimating the proportion of the population which is sterile were also developed. These methods can be applied to other types of outcomes, such as the number of pregnancies required in order to achieve a live birth. Preliminary investigations of appropriate designs (prospective or retrospective) for the collection of such data were begun, and this work will continue. Preliminary research was also begun into the assessment of fertility following an intervention, which might be either a treatment or a suspect exposure.

2) Some of the statistical methods which are currently used to analyze spontaneous abortion (miscarriage) data were studied. Spontaneous abortion data are plagued by various types of biases which are caused by the interaction between heterogeneity of risk from woman to woman and the possibility of accurate control of fertility by the women. Women who are at increased risk of abortion tend to have more pregnancies than do women who are lower risk in an effort to compensate for the abortions which occur and achieve a certain desired family size. Current techniques for handling abortion data are generally of two types: either they ignore the potential problems altogether, or they attempt (usually unsuccessfully) to separate a group of women into "normal" and "habitual aborter" subgroups. It was demonstrated that both of these classes of techniques will lead to biases in the estimation of rates and thus to incorrect conclusions. Indeed, it was shown that some techniques can cause a group with a higher abortion rate than the control group to appear to have a lower rate. It is hoped that understanding the problems with existing methods will

lead to the development of better techniques, which would allow the assessment of whether suspect agents caused an increase in abortion rates.

3) Statistical methods have previously been developed to assess the reflex relationship between heart rate and respiration (Biometrics, 1984, below). The potential application of these methods to screening for pre-symptomatic autonomic neurotoxicity, and as a possible marker for babies at risk of SIDS (sudden infant death syndrome), is now being studied. A pilot study using tapes of heart rate and respiration has been completed; it yielded two workable methods for summarizing and analyzing the data. One of these methods involves computing the amplitude of the cross-correlation between detrended instantaneous heart rate and the respiratory signal. The other is a direct extension of the vector technique already developed for screening for neuropathy in diabetic patients, in work cited below (Biometrics, 1984). A case-control study will be done, comparing prospectively gathered recordings from babies who later died of SIDS to surviving babies matched for age, sex, gestational age at birth, and respiratory rate. It is hoped that this study will shed light on possible environmental and developmental mechanisms involved in SIDS.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Although the Institute relies heavily on animal data from the laboratory, we are ultimately concerned about human health, and thus epidemiologic data are potentially of particular importance. Unfortunately, epidemiologic data are as a rule, much more complex than laboratory data, primarily because the former is observational in nature rather than experimental. Many of the factors which in the laboratory can be controlled experimentally, can only be controlled statistically when studies involving free-living humans are analyzed. With observational data, the possibility of a range of biases distorting the results is an ever-present worry, so that careful statistical analysis of the data is essential. The development and evaluation of appropriate statistical tools is important if epidemiologic data collected by the Institute or by other organizations are to yield their full value and be correctly interpreted.

PUBLICATIONS

Weinberg, C.R.: Applicability of the simple independent action model to epidemiologic studies involving two factors and a dichotomous outcome. American Journal of Epidemiology, in press.

Weinberg, C.R.: On pooling across strata when frequency matching has been followed in a cohort study. Biometrics 41:117-128, 1985.

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Weinberg, C.R. and Pfeifer, M.A.: An improved method for measuring heart-rate variability: assessment of cardiac autonomic function. Biometrics 40:855-861, 1984.

Yanagawa, T.: Case-control studies: assessing the effect of a confounding factor. Biometrika 71:191-194, 1984.

Yanagawa, T., Kasagi, F., and Yoshimura, T.: A method for estimating incidence rates of onchocerciasis from skin-snip biopsies with consideration of false negatives. Biometrics 40:301-311, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 40005-08 SBB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Methodology and Analysis of Mutagenesis Testing Data

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Barry H. Margolin	Mathematical Statistician	SBB	NIEHS
Others:	Ken Risko	Mathematical Statistician	SBB	NIEHS
	Doug Simpson	Mathematical Statistician	SBB	NIEHS
	Randy Tobias	Mathematical Statistician	SBB	NIEHS
	Errol Zeiger	Supervisory Microbiologist	CGTB	NIEHS

COOPERATING UNITS (if any)

Cellular Genetics and Toxicology Branch, TRIP

LAB/BRANCH

Statistics and Biomathematics Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

2.9

PROFESSIONAL:

2.9

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

During the last decade the science of genetic toxicology has experienced dramatic growth in its volume of experimentation, its variety of assays, and its level of public awareness. This growth, in all its dimensions, is attributable to the ability of these short-term tests to detect, rapidly and relatively inexpensively, environmental agents that are genotoxic. These agents are thought to be implicated in such diverse human health problems as cancer, aging and birth defects. Scientific research in genetic toxicology, however, is far from mature; much remains to be achieved in terms of understanding the precise implications of results from such tests for the assessment of risks to human health. To this end, it is important that there be objective methods of analysis of assay system test results, so that subjective assessments do not significantly interfere with the evaluation of the relative merits of short-term tests. Thus, development of appropriate statistical techniques for the analysis of data arising from short-term assays remains the primary goal of this project. In addition to the development of statistical analyses, an increasing amount of attention has been paid to: (i) methods for meaningful assay validation and, (ii) the exploitation of large databases for the assessment of interlaboratory and interassay concordance. In response to the heightened interest in in vivo mechanisms of genetic toxicity, this project has been broadened to include study of a number of in vivo tests. Efforts have also been expended to explore those issues that are uniquely characteristic of genetic toxicity studies of human subjects, an area of increasing research activity.

PROJECT DESCRIPTION

METHODS EMPLOYED: A mixture of mechanistic and empirical modeling, together with statistical data analytic and Monte Carlo procedures, is the main methodological approach taken toward these studies. The following large computerized databases for short-term test data have been constructed and maintained: (a) results of the Ames Salmonella/microsome Test, a bacterial assay that is preeminent among short term tests for mutagenicity, (b) in vitro cytogenetics data for sister chromatid exchanges and chromosome aberrations, and (c) International Program on Chemical Safety data from two Collaborative Studies on Short-Term Tests. All three have contributed substantially to the continued productivity of this project. Via these databases, much effort has been expended to subject common statistical and biological assumptions to empirical checks for validity.

MAJOR FINDINGS AND PROPOSED COURSE: (1) Statistical analyses of the IPCS Part I In Vitro Study data strongly support the study's conclusion that "Significant differences exist among individual investigators conducting nominally identical assays Inadequate test protocols clearly contributed to some assays appearing to be insensitive." (2) In a joint effort with CGTB, standard protocols for in vitro cytogenetics testing with Chinese hamster ovary (CHO) have been developed, and have undergone extensive analysis and validation. For the analysis of data generated with these protocols, it has been shown that a proposed trend test for SCE data is more sensitive than four other applicable statistical procedures, including the chi-square and Dunnett tests. Similar results hold for the proposed trend test analyses of chromosome aberration data. While the scoring for SCE's of 50 CHO cells/dose point provides adequate sensitivity, the customary scoring of 100 cells/dose point for chromosome aberrations is too insensitive to detect weak, environmental clastogenic agents. An increase to 200 cells/dose point seems worthy of serious consideration. (3) A general recursive strategy has been developed to test nonparametrically for an increasing dose-response relationship when a downturn in response at high doses is possible because of competing risks. A specific implementation involving the Jonckheere-Terpstra test has been studied extensively, and appears to provide a very good alternative to tests constructed solely for monotone trend. The latter exhibit a substantial loss of power when there is a downturn in the dose response at high doses because of competing risks, e.g., cellular toxicity. (4) A common model in radiation and chemical mutagenesis describes the probability of a mutagenic event as a function of dose as follows:

$$p(D) = [1 - \exp(-\alpha + \beta D)] \exp(-\gamma D).$$

Extensive Monte Carlo studies indicate that the likelihood ratio test and the normal test based on the ratio of the maximum likelihood estimate of β to its standard deviation obtained from the Fisher Information matrix are not well behaved. A modification based on the incorporation of a pretest for $H_0: \gamma=0$ improves matters substantially.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The development of methods of statistical analysis for in vitro cytogenetics assays, together with past and present work on the analysis of Ames Test data, provides NTP with reliable, satisfactory methods for the analysis of the two genetic toxicity assays that are currently key to an environmental mutagenesis program. These statistical procedures will aid appreciably in the study of interlaboratory and interassay concordance. Equally important, the results of employing validated, sensitive statistical procedures to short-term tests should improve the process of assessing their predictive value vis a vis the chronic rodent carcinogenicity bioassay. A further benefit of this project is that the methodological results obtained are frequently of interest to biometricians in other areas of research.

PUBLICATIONS

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Margolin, B.H. and Shelby, M.D.: Sister chromatid exchanges: A reexamination of the evidence for sex and race differences in humans. Environmental Mutagenesis, Supplement 4: 63-72, 1985.

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Ashby, J., de Serres, F.J., Draper, M., Ishidate, M. Jr., Margolin, B.H., Matter, M., and Shelby M.: Overview and conclusions of the study. In: Report of the International Program on Chemical Safety collaborative study on in vitro assays. Elsevier/Science Publishers Amsterdam, 117-174, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 41001-11 SBB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Risk Assessment Methodology Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	David G. Hoel	Chief	SBB	NIEHS
Others:	Michael D. Hogan	Mathematical Statistician	SBB	NIEHS
	Chris. J. Portier	Mathematical Statistician	SBB	NIEHS
	Eiji Yamamoto	Visiting Fellow	SBB	NIEHS

COOPERATING UNITS (if any) Department of Biostatistics, School of Public Health, University of North Carolina; Developmental Biology Division, Health Effects Research Laboratory, EPA; Toxicology Research and Testing Program, NIEHS; Laboratory of Biochemical Risk Analysis, BRAP.

LAB/BRANCH

Statistics and Biomathematics Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

1.5

PROFESSIONAL

1.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project is concerned with the development of statistical and mathematical methodology useful in the assessment of risks associated with exposures to potentially hazardous environmental and occupational agents. A major focal point is the generation of improved statistical techniques for estimating adverse human health effects from laboratory animal data, with particular emphasis being placed on dose-response modeling, low-dose extrapolation and extrapolation of toxicologic responses across species. Consideration is also given to the modeling of epidemiologic data in the risk assessment process. Current research efforts are concerned with the evaluation of a proposed procedure for estimating carcinogenic potency, with the investigation of factors that can affect the correlation of carcinogenic potency between mice and rats, with the assessment of the impact of litter effects on dose-response modeling in teratology, and with the extension of dose-response curves beyond the experimental dose range through the use of molecular dosimetry.

METHODS EMPLOYED: In addressing various methodological issues that are involved in the assessment of human health risks, a number of different statistical and mathematical techniques are employed. For example, existing data bases are used to make empirical observations and to assess the reliability of different statistical and biomathematical models. Models are constructed and their properties are studied by both simulation and analytical methods. In addition, experimental data is generated and used to investigate issues such as the biologically effective dose in dose-response modeling and extrapolation.

MAJOR FINDINGS AND PROPOSED COURSE: A number of different measures of carcinogenic potency have been generated and used by investigators to make "standardized" comparisons of different chemicals, test species, strains, routes of exposure, etc. Among those measures that have received a great deal of attention is the TD_{50} index proposed by Peto and his collaborators. While the TD_{50} appears to have many of the desirable features of the more traditional LD_{50} index used in standard laboratory bioassays, its usefulness as a measure of potency has not been extensively investigated. Therefore, the bias and estimated variance of this measure are being evaluated for a variety of dose-response patterns commonly encountered in carcinogenesis screening studies. Furthermore, since the most appropriate determination of relative carcinogenic potency should depend on the shapes of the dose-response curves for the chemicals or species being compared as well as on their estimated TD_{50} values, a recently developed procedure for differentiating between linear and non-linear curvature is also being assessed. Simulation and data-analytic approaches are being used to study the sensitivity and specificity of this procedure.

The high correlation of carcinogenic potency observed between mice and rats was evaluated for a large number of chemicals using data from NCI/NTP carcinogenesis screening studies and related experiments in the published literature. This evaluation indicated that there was also a high correlation between the maximum dose levels tested (MTD) for mice and rats on a mg/kg/day basis, and that the estimated carcinogenic potency (TD_{50}) was statistically restricted to a 30-fold range about the MTD. Since the MTDs in the data base varied over several orders of magnitude, it follows statistically that the carcinogenic potencies would be highly correlated. Thus, a great deal of care needs to be exercised when conducting and interpreting correlational studies of carcinogenic potency based on the TD_{50} .

The fitting of dose-response models to teratology data involving littermates in order to generate estimates of teratogenic risk is a potential alternative to the safety factor approach to risk estimation. The models that have been most frequently proposed for characterizing teratogenesis dose-response functions are those commonly employed in cancer risk estimation. In this modeling process litter effects are typically ignored, and the models are simply fit to the proportion of affected fetuses or litters observed at each dose level. To evaluate the possible impact of litter effects on dose-response modeling, a simulation study was conducted using the beta-binomial distribution to introduce varying degrees of intra-litter correlation. Results indicate that failure to properly account for intra-litter correlation may affect the estimated variances associated with model parameter estimates and, in some instances, introduce bias into the estimation process itself.

As a result of a collaboration with Dr. Birnbaum of the TRTP, an existing contractual investigation of the pharmacokinetics of inhaled substances has been modified to include a number of dosimetry studies of benzene exposure. Data generated under this contract will be used to make detailed species and route of exposure comparisons (e.g., to see if there is a metabolism saturation difference by exposure route). Dose-response relationships will also be evaluated for a number of different parameters such as tissue distribution of key benzene metabolites, DNA adducts, and cytogenetic effects. This evaluation should allow the benzene dose-response relationship to be extended beyond the normal experimental range and, therefore, should enhance the extrapolation of low-dose benzene exposures to the human situation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Risk assessment, particularly quantitative risk assessment, is an increasingly important component of the regulatory process concerned with protecting humans from the adverse health effects of occupational/environmental exposures. As our ability to characterize or quantify the actual risk associated with a given exposure situation is enhanced, we will be better able to determine whether or not there is a need for regulatory action and to evaluate the effectiveness of any such action (or other risk management option) that is taken. Therefore, mathematical or biostatistical research that reduces the uncertainty associated with the risk assessment process or that broadens the scientific basis of the process has obvious significance for and benefit to the biomedical community and the general public.

PUBLICATIONS

Bernstein, L., Gold, L.S., Ames, B.N., Pike, M.C., and Hoel, D.G.: Some tautologous aspects of the comparison of carcinogenic potency in rats and mice. Fundamental Appl. Toxicol.: 5:79-86, 1985.

Hoel, D.G. Mathematical dose-response models and their application to risk estimation. In: Vouk, V.B., Butler, G.C., Hoel, D.G., and Pekall, D.B. (Eds.): Methods for Estimating Risk in Humans and Chemical Damage in Nonhuman Biota and Ecosystems, SCOPE 1985/SGOMSEC 2, Chichester, NY, Brisbane, Toronto, Singapore, J. Wiley and Sons, 1985, pp. 347-359.

Hoel, D.G.: The Incorporation of Pharmacokinetics in Low-Dose Risk Estimation. In: Clayson, D., Krewski, D. and Munro, I. (Eds.): Toxicological Risk Assessment. Florida, CRC Press. (in press).

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Hoel, D.G. and Jennrich, R. I.: Life table analysis with small numbers of cases: An example - multiple myeloma in Hiroshima and Nagasaki. J. Stat. Computation and Simulation 20: 311-322, 1985.

Hoel, D.G. and Preston, D. L.: Dose-response in radiation carcinogenesis: Human studies. In Castellani, A. (Ed.): Epidemiology and Quantitation of Environmental Risk in Humans from Radiation and Other Agents: Potential and Limitations, London, Plenum Press (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 44002-09 SBB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical Modeling of Molecular Phenomena

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Norman L. Kaplan Research Mathematician SBB NIEHS

Others: Charles H. Langley Research Chemist SBB NIEHS
Thomas Darden Staff Fellow SBB NIEHS
Richard Hudson Staff Fellow SBB NIEHS

COOPERATING UNITS (if any)

Laboratory of Animal Genetics, LRDT

LAB/BRANCH

Statistics and Biomathematics Branch

SECTION

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The purpose of this research project is to develop and study mathematical models for certain biological phenomena at the molecular level. Current work has focused on (a) developing new estimates of the rate of recombination in finite Mendelian populations and studying the statistical properties of existing estimates, (b) analyzing the statistical properties of a general stratified sampling scheme for studying genetic variation in natural populations, (c) studying certain selective models which describe the evolution of a family of transposable elements and (d) applying existing methods in computational chemistry to help explain toxicological outcomes of environmental chemicals.

PROJECT DESCRIPTION

METHODS EMPLOYED: Two approaches were used to study the models proposed. The analytic approach exploited existing mathematical theory to prove useful properties about the models. Existing results were used from the theories of Markov processes and diffusion processes. Many of our results led to large systems of recursive equations which could only be solved numerically. Hence, computer intensive methods were needed. In certain cases the models under consideration were too complicated to study analytically and so sophisticated simulation methods were used instead. Simulations also proved useful in verifying the analytic results.

MAJOR FINDINGS AND PROPOSED COURSE: (a) Work is continuing on the problem of estimating the rate of recombination for a multilocus infinite-allele model (i) the first part of this work was to study the statistical properties of existing estimates. One often used estimate of linkage disequilibrium (a quantity which is closely related to the rate of recombination) was shown by simulation to be inaccurate unless the frequencies of the alleles at the different loci were taken into account. In particular, it was found that large estimates of linkage disequilibrium did not provide a good basis for rejecting a neutral model. (ii) A fundamental problem of estimating the rate of recombination is that, R , the number of recombination events in the history of a sample of sequences cannot be estimated from the data. We therefore studied a related quantity R_M which is the number of recombination events which can be parsimoniously inferred from the sample data. Some analytic properties of R and R_M were determined, and by simulation it was shown that R_M is often less than R . Hence, using R_M instead of R to estimate the rate of recombination leads to biased estimates. Simulation methods were used to study the extent of this bias. (iii) In the course of this investigation, it was necessary to calculate for an m -loci model the variance of the number of segregating sites in a sample of arbitrary size. A method based on the genealogy of the sample rather than a diffusion approximation was developed. This method was also used to calculate m -loci homozygosity. (b) Since several methods of varying technical difficulty now exist for determining genetic variation at the molecular level (e.g. electrophoresis, restriction enzyme mapping and DNA sequencing) a stratified sampling approach for surveying DNA variation in a natural population is attractive. Methods have been developed for analyzing this type of data assuming that the initial sample data (e.g. electrophoretic data) can be described by a neutral infinite allele model and the sub-sample data (e.g. sequence data) by the neutral infinite site model. Work is now underway to apply the techniques used to derive these results to other related questions. In particular, we are studying methods for making inferences about the genealogy of a sample when several different kinds of mutational data are available. This work also led to an investigation of the connection between the genealogy of a sample and the calculation of identity coefficients. As an application we developed a general scheme for calculating identity coefficients for models of gene conversion in multigene families. (c) Work is still ongoing in the study of selective models for the evolution of a family of transposable elements. At this stage we are investigating selective models related to recombination.

PROJECT DESCRIPTION

(d) The low energy vacuum state conformations of DES and the Z and E forms of pseudo-DES have been explored theoretically using empirical, semi-empirical and ab-initio methods, and compared with the results of x-ray diffraction analysis of the solid state for these three compounds. A simple model of the PCB receptor using porphyrin has been tested for its ability to reproduce experimental binding correlations using molecular mechanics binding energy computations together with various molecular graphics techniques. Finally, the thyroxine binding protein albumin, which has a known 3-dimensional structure and which is conjectured to play a role in the toxicity of PCB's and Dioxin, is being examined in an attempt to correlate the binding of PCB's, dioxin and polybrominated naphthalenes with theoretically predicted binding affinities.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: With the development of new techniques for studying DNA variation, fundamental questions about the consequences of high observed levels of genetic variation on the future of an individual can begin to be addressed. An essential component of this endeavor is the development of statistical methods for analyzing the ever increasing flow of molecular data. These statistical methods are based on an underlying model and so it is important to make the model as realistic as possible; e.g., incorporating mutation and recombination. Good estimates of mutation and recombination rates are important both for theoretical reasons (e.g., understanding molecular evolution in general) and for practical reasons (e.g. calculating diagnostic rates in prenatal diagnoses of genetic diseases using genetic markers).

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 ES 45001-05 SBB
PERIOD COVERED October 1, 1984 to September 30, 1985		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Experimental Design and Data Analysis Methodology for Animal Experiments		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Joseph K. Haseman Research Mathematical Statistician SBB NIEHS Others: Christopher J. Portier Mathematical Statistician SBB NIEHS Gregg E. Dinse Senior Staff Fellow SBB NIEHS Walter W. Piegorsch Mathematical Statistician SBB NIEHS		
COOPERATING UNITS (if any)		
LAB/BRANCH Statistics and Biomathematics Branch		
SECTION		
INSTITUTE AND LOCATION NIEHS, NIH, Research Triangle Park, North Carolina 27709		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) This project is concerned with statistical methodology issues involved in the design, analysis, and interpretation of laboratory animal experiments, with particular emphasis on two-year carcinogenicity studies. Experimental design issues include the use of the maximum tolerated dose (MTD) and the possible utilization of multiple concurrent control groups in carcinogenicity studies. Research in the area of data analysis has focused on the use of logistic regression techniques to analyze non-fatal tumors, modeling tumor onset distributions, and the assessment of synergistic effects in carcinogenicity testing. Statistical issues affecting the interpretation of carcinogenicity studies include evaluating overall false positive rates and assessing the biological significance of correlated tumor responses.		

PROJECT DESCRIPTION

METHODS EMPLOYED: The evaluation of existing experimental designs and analytical procedures and the development of new methodologies require the use of a number of different mathematical/statistical techniques including mathematical modeling, non-linear optimization, Monte Carlo simulation, maximum likelihood estimation, and regression analysis. With regard to application of these techniques, the computerized data base of tumor incidences and other information from carcinogenicity studies carried out by the National Toxicology Program (NTP) were utilized in a number of statistical evaluations.

MAJOR FINDINGS AND PROPOSED COURSE: Results of major research projects in the areas of experimental design, data analysis, and interpretation of experimental results are summarized below.

I. Experimental Design

One recently suggested design modification in long term carcinogenicity studies is the utilization of multiple concurrent control groups. To investigate this issue further, tumor incidence data from 18 recently completed studies involving male and female mice and rats were examined to determine if the frequency of significant ($p < 0.05$) pairwise differences between the two concurrent control groups employed in these experiments exceeded chance expectation. Although marked study-to-study variability was observed for some tumors, no evidence of extra-binomial within-study variability was found. The total number of observed significant ($p < 0.05$) paired-control differences was virtually identical to what would be expected from the usual binomial model assumptions; similarly, the corresponding overall observed and expected false positive rates were essentially the same. Hence, these data provide no evidence to suggest a need for multiple concurrent control groups.

Dose selection is a critical issue in the design and evaluation of laboratory animal carcinogenicity studies, and the current practice of utilizing the maximum tolerated dose (MTD) was investigated. Data from 52 NTP studies indicated that while the MTD may have been exceeded in certain studies using the gavage route of administration, there was little evidence that this was a problem in NTP studies using the dietary (feed) route of exposure. These data also indicated that more than two-thirds of the carcinogenic effects detected in feeding studies (involving many different organ sites and tumor types) would have been missed had the high dose (MTD) not been used, and decisions regarding carcinogenicity been based on the responses observed in the low dose (1/2 MTD) group. The inherent insensitivity of laboratory animal studies for detecting weak to moderate carcinogenic responses also argues against reducing the highest dose level.

The NTP recently modified its experimental protocol for histopathology evaluation. First, controls and high dose animals receive a complete histopathology examination. Then, lower dose groups are examined for those target organs identified in the initial examination. This experimental design modification has the potential to change the size and power of the statistical procedures used to assess differences in tumor incidence. Preliminary results indicate that the magnitude of this change is small.

II. Data Analysis

The problem of assessing possible interactions among chemical exposures is important, both for regulating acceptable exposure levels and for understanding their possible mechanism of action. The carcinogenic potential of a test compound may be enhanced (or inhibited) when it is administered in combination with another substance. Various procedures for assessing interaction (i.e., synergism or antagonism) for tumor incidence data were evaluated. Finney's null model of simple, independent action was adopted, and size and power was examined via large-scale simulations for various proposed statistics under a variety of model parameterizations. Among the procedures considered, the method of Wahrendorf et al. (Biometrics, 37: 45-54, 1981) appeared to perform best.

Tumor prevalence, lethality and mortality are three important descriptors of the development and course of a tumor. Parametric and nonparametric maximum likelihood estimators were derived for these functions. The proposed estimators incorporate sacrifice data and full or partial information on cause of death. No assumptions are made about the degree of tumor lethality or the distribution of survival times.

Most current carcinogenesis studies only indicate whether or not a particular tumor is present at death and do not provide information on cause of death for individual animals. Ignoring the possibility of increased mortality in animals which have a particular tumor can lead to biases in the quantitative estimates of risk. Statistical methods are being developed and studied which account for the increased risk of dying in animals with a tumor using stochastic models to describe the progression from tumor onset to death with a tumor. These methods do not require cause of death information.

One factor that has traditionally inhibited the optimal analysis of tumor incidence data is the lack of available cause of death information on an individual animal basis. Methodology being developed by the SBB allows a statistical analysis to be carried out when cause of death information can be obtained for only a subset of animals and even when cause of death information is unavailable for all animals.

Parametric models for the distribution of tumor onset times in historical control animals are being studied. Various methods are being used to estimate the tumor onset distribution and the degree of lethality for the target sites commonly studied in carcinogenesis experiments to further our understanding of the development and progression of these lesions.

III. Data Interpretation

Chemicals often appear to both increase and decrease tumor incidences in laboratory animal carcinogenicity studies. Thus, it is important to identify any consistent associations in tumor response to aid in the overall evaluation and interpretation of a chemical's carcinogenic potential. Data from recent NTP studies were found to provide additional support to the previously-reported negative association between liver tumor incidences and leukemia rates in carcinogenicity experiments utilizing Fischer 344 rats.

The impact of basing a statistical analysis on total tumors or on all malignant tumors (i.e., the proportion of tumor-bearing animals or the proportion of animals with malignant neoplasms) was studied for NTP experiments in Fischer 344 rats and B6C3F1 mice. It was found that because of the high spontaneous tumor frequencies in these animals, a chemically-related tumor increase could easily be masked by basing an analysis on total tumors or on all malignant tumors. Similar results were found even if certain high spontaneous incidence tumors were excluded from the evaluation. Thus, analyses based on site-specific tumor incidences are preferable.

Further research in this area will continue to focus on these and other statistical issues in carcinogenicity testing, to improve the experimental design and data analysis of these experiments and to further our ability to interpret the biological significance of the results of these studies.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The NTP carcinogenesis testing program is the federal government's primary means of screening compounds for carcinogenic potential. In addition, it is one of the main sources of data for assessing human cancer risk. Therefore, any methodological development that improves its effectiveness is important to both the Institute and to the biomedical community in general.

PUBLICATIONS

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Dinse, G.E.: An alternative to Efron's redistribution-of-mass construction of the Kaplan-Meier estimator, The American Statistician (in press).

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Larson, M.G. and Dinse, G.E.: A mixture model for the regression analysis of competing risks data, *Journal of the Royal Statistical Society, Series C (Applied Statistics)* (in press).

Portier, C.J.: Design of animal carcinogenicity experiments: Dose allocation, animal allocation and sacrifice times, in *Proceedings of the Symposium on Long-Term Animal Carcinogenicity Studies: A Statistical Perspective* (in press).

Portier, C.J. and Hoel, D.G.: Design of the chronic animal bioassay for goodness-of-fit to multistage models, *Fundamental and Applied Toxicology* 4: 949-959, 1984.

Racine-Poon, A.H. and Hoel, D.G.: Nonparametric estimation of the survival function when cause of death is uncertain, *Biometrics* 40: 1151-1158, 1984.

Yanagawa, T. and Hoel, D.G.: Use of historical controls for animal experiments. *Environmental Health Perspectives*, (in press).

LAWRENCE BERKELY LABORATORY/UNIVERSITY OF CALIFORNIA
Berkeley, California 94620

(Y01-ES-10066)

TITLE: Quantitative Species Extrapolation in Carcinogenesis

CONTRACTOR'S PROJECT DIRECTOR: David G. Hoel, Ph.D., Chief;
Michael D. Hogan, Ph.D., Mathematical Statistician
Biometry and Risk Assessment Program

COLLABORATING INSTITUTE: Department of Energy

DATE CONTRACT INITIATED: April 1, 1981

CURRENT ANNUAL LEVEL: \$325,000 (Estimated)

PROJECT DESCRIPTION

OBJECTIVES: The quantitative assessment of factors that may lead to differential species responses to a carcinogenic exposure and the development of procedures for extrapolating carcinogenic risk projections across species have been hampered by the very limited availability of epidemiologic data suitable for quantitative interspecies comparisons. Therefore, this project was initiated in part to develop a large-scale laboratory animal database for making non-human interspecies comparisons to evaluate quantitatively the impact that various factors have on the variability in species responses observed in long term carcinogenic screening studies.

METHODS EMPLOYED: A set of acceptability criteria covering minimum sample size, use of concurrent controls, duration of experiment, length of exposure, route of administration, etc. were developed for evaluating studies reported in the literature or in NCI/NTP technical reports and deciding which should be abstracted into a computerized database. Information from this database has in turn been used to address a number of specific issues that bear directly on the quantitative extrapolation of cancer risk estimates across species. All intra and interspecies comparisons have been based on the TD₅₀ i.e., the dose rate in mg/kg/day that, when administered chronically over the test species' lifetime, will reduce the probability of remaining tumor-free over the study interval by a factor of two.

MAJOR FINDINGS AND PROPOSED COURSE: During the present reporting period: (1) The carcinogenic potency data-base was updated to incorporate all published studies through 1983 that satisfied the established inclusion criteria. (2) The carcinogenic potency database (through 1981) and a related article on the use of TD₅₀ index as a measure of carcinogenic potency were published as Volume #58 (December, 1984) of Environmental Health Perspectives. (3) Collaborative research was initiated to develop a summary potency index for chemicals tested in more than one sex, species, strain, or route of exposure, or for chemicals

that induced tumors at more than one site. A number of different procedures have been proposed, and their relative strengths and limitations are being assessed. (4) Research continued on the evaluation of the reproducibility of potency, pathology, and positivity for chemicals tested more than once in the same species, strain, sex and by the same exposure route. (5) The study of the potential association between carcinogenic potency and various parameters of rodent tumor pathology such as tumor site, whether tumors were induced at multiple sites, whether tumors were fatal or incidental, and whether or not tumors metastasized was completed.

Consideration is currently being given to a variety of projects including the expansion of the database by the addition of experimental results from new, recently published sources, the further exploration of the relationship between toxicity and carcinogenic potency, and the evaluation of the relevance/utility of the rodent TD₅₀ index in terms of human exposure levels.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Given our increasing reliance on laboratory animal data to generate quantitative estimates of human cancer risk, there is a clear need to improve our ability to extrapolate cancer screening results across species. Therefore, research addressing this issue is certainly in keeping with the research goals of the Institute.

(N01-ES-1-5009)

TITLE: Establishment and Maintenance of an International Register of Persons
Exposed to Phenoxy Acid Herbicides and Contaminants

Contractor's Project Director: Rodolfo Saracci, M.D.

Project Officer (NIEHS): David G. Hoel, Ph.D., Director
Michael D. Hogan, Ph.D., Mathematical Statistician
Biometry and Risk Assessment Program

Date Contract Initiated: July 20, 1981

Current Annual Level: No additional funds obligated in FY'85

PROJECT DESCRIPTION

OBJECTIVES: To establish and maintain, under the auspices of the International Agency for Research on Cancer, an international registry of cohorts/individual workers exposed to phenoxy acid herbicides and their contaminants. This Registry will eventually be used as a database for various epidemiologic studies focusing on: (1) retrospective and prospective cohort mortality with analysis for cancer and other causes; (2) cancer incidence in areas where population-based cancer registry data are available for linking; and (3) special clinical and laboratory follow-up investigations.

METHODS EMPLOYED: (1) Preparation of a draft standardized protocol to be used by all participating countries in presenting their data to the registry is nearing completion. Upon completion it will be circulated among project collaborators for comment. (2) Collaborators have been contacted to solicit copies of protocols and data forms being used to study exposed cohorts and to obtain responses to a questionnaire on the types of data available in their countries (e.g., nature of exposures, completeness of follow-up of study subjects, ability to code causes of death other than the underlying cause, etc). (3) Site visits have been made to some of the major collaborating centers by the project epidemiologist to obtain first-hand, detailed knowledge about various issues such as the definition of exposure that are pertinent to the establishment of the registry.

MAJOR FINDINGS AND PROPOSED COURSE: (1) A directory of the world's major producers of chloro phenoxy acid herbicides and chlorophenols has been prepared and will be used to identify additional countries that might participate in the registry as well as additional data resources for collaborating countries. (2) Initial collaborator responses to the questionnaire on data availability indicate that most major exposures of interest are likely to be represented and that high follow-up rates are anticipated. (3) Data generated to date by collaborating countries suggest that the total number of workers with definite phenoxy acid herbicide exposure that will be enrolled in the registry could significantly exceed initial estimates.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Because of the general concern that exists about the potential health risks associated with exposure to phenoxy acid herbicides such as 2,4,5-T and related contaminants, there is a definite need for additional human data on exposed cohorts to create a database for the epidemiologic evaluation of these risks.

TITLE: Statistical Analysis of Bioassay Data

CONTRACTOR'S PROJECT DIRECTOR: Steve Seilkop

PROJECT OFFICER (NIEHS): Joseph K. Haseman, Ph.D.
Research Mathematical Statistician
Statistics and Biomathematics Branch, BRAP

DATA CONTRACT INITIATED: July 31, 1981

CURRENT ANNUAL LEVEL: \$ 287,126

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this contract are to provide statistical and computational expertise and resources to summarize, analyze, and aid in the interpretation of data from various NTP experiments. These investigations consist of carcinogenesis experiments, pre-chronic studies, and certain other "special studies" with laboratory animals.

MAJOR FINDINGS AND PROPOSED COURSE: Over the past year, PRI has provided support to the NTP in the statistical analysis of data from its carcinogenesis bioassay program. Data from approximately 20 pre-chronic studies used for selection of doses in chronic studies were computerized and statistically analyzed. Organ and body weight, hematology, clinical chemistry, and histopathology findings were statistically evaluated and interpreted in reports to the NTP. PRI is also responsible for a continuing study of viral infections in the bioassay program's sentinel animals and prepares semi-annual reports on viral incidence. As well as supporting the carcinogenesis bioassays, the contract provides statistical assistance in the analysis of data from NTP studies of chemical effects on reproduction and fertility in laboratory mice. Over the past year, PRI has prepared summary statistical reports for over 20 of these studies.

A major activity of the contract has been to build accessible data bases for both pre-chronic and reproductive study data. Using the pre-chronic data base, PRI recently prepared a report on the distribution of historical control data for body and organ weights, hematology, and clinical chemistry. Currently, PRI is also utilizing these resources to investigate sources of systematic variation and to provide insight into improving methods of statistical analysis.

Through this contract PRI is becoming increasingly involved in the analysis of data from NTP chronic studies. In addition to maintaining existing statistical software on the Toxicology Data Management System, it is developing new software to reflect current statistical methods in the analysis of tumor incidence data. Recently, for example, a logistic regression program was added to TDMS.

Over the next year the contract will continue to provide statistical support in the analysis of NTP's pre-chronic, chronic, sentinel animal, and reproduction and fertility studies. Increased effort will be devoted to enhancement of TDMS statistical software and the study and use of historical data from pre-chronic studies.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE NTP: This contract provides the statistical support necessary to effectively analyze large volumes of pre-chronic, chronic, and special studies data generated from various NTP laboratory animal studies.

EXTRAMURAL PROGRAM

CENTERS PROGRAM

The NIEHS supports nine Environmental Health Science Centers and three Marine and Freshwater Biomedical Centers. The purpose of the Centers program is to provide core support to select institutions with major research efforts focusing on environmental health. The goal is to provide a base of support for scientists in fields relating to the mission of the Institute to allow them to plan long-term research activities investigating questions relating to environmental health sciences thereby producing new knowledge to allow better assessments of potential hazards to human health in the environment. These Centers also become resources to the Institute in that their individual or combined expertise can be brought to bear on health issues of immediate importance to the general public. In addition, the Institute stimulates the scientific exchange of information and plans at formal semi-annual Center Directors' Meetings and informally throughout the year.

A new thrust in the Centers' Program was announced this year - the Specialized Centers of Research (SCOR) program. SCORs are full-support (core plus research) Centers which focus on a specific area of interest to the Institute. Three SCOR thrusts were announced as follows:

1. CNS Neurotoxicology: To utilize new cell culture techniques to isolate specific CNS cells and study the effect of foreign chemicals on their function.
2. Genetic Toxicology: To specifically identify DNA adducts resulting from doses of environmental chemicals and relate these adducts to exposures and metabolism.
3. Marine and Freshwater Biomedical Sciences: To utilize marine models for studying the effects of human exposures to environmental contaminants. This program will replace the Marine and Freshwater Biomedical Core Center grants mentioned above.

A listing of extant Core Centers and a brief summary of activities at each follows.

University of California, Berkeley

The Center at Berkeley has been concerned with basic and applied aspects of the integrity of DNA as related to public health. Methods have been developed for examining DNA damage in individual humans or animals by analyzing particular DNA damage products excised from DNA and excreted in urine. These studies are being done to determine if oxidative DNA damage is a major determinant of both cancer and aging.

University of Rochester

The Environmental Health Sciences Center at Rochester focuses its main efforts on the toxic consequences of metal exposure. Metals are so ubiquitous in nature, in our industrial environment and in our body tissues, that they continue to evoke questions about hazards to health. Some of these are

well-recognized, but at high exposure levels. The Rochester Center sees its task as the prediction of the health consequences of exposure to low levels. Some hazards are only beginning to make themselves known, partly because of their subtlety, and partly because metals are being encountered in new, exotic forms. The semiconductor industry, for example, uses more organic metal compounds that, from what we already know of similar agents, are probably quite toxic. Scientists at the Rochester Center have been especially active in trying to evaluate the health threats of methylmercury, an organic form of mercury.

Vanderbilt University

The Center in Molecular Toxicology at Vanderbilt University conducts research in several areas related to understanding toxic phenomena at the chemical and biochemical levels. For example, monoclonal antibodies to marker proteins are being used to detect presences of the marker protein in rat liver cells freshly transformed to malignancy by chemical carcinogens and the interaction of potentially toxic metals with DNA and chromatin proteins is being investigated. Other studies focus on the development of new chelating agents which can be used as antidotes for heavy metal toxicity. The chemistry of the interaction of the carcinogen ethylene dibromide with DNA has been elucidated and methods are being developed to assess the dangers of the compound. A number of toxic, natural products found in foodstuffs have been characterized and are being studied further. The molecular biology related to DNA repair processes is being studied. Investigators are also characterizing human enzymes which metabolize xenobiotic chemicals and vary among individuals. Probes for these enzymes are being developed to assess the role of host factors in individual risks.

Harvard University

The Harvard University Center provides support for a wide range of studies designed to understand the mechanisms of action of environmental agents related to man's health. The primary human disease focus is upon cancer, chronic lung disease, neurobehavioral effects, and effects of agents on the reproductive system. Emphasis is placed upon (a) occupational hazards with specific interest in carcinogens, pulmonary pathogenic substances, and neurotoxic compounds, (b) community air pollution, (c) cigarette smoking, (d) radiation, (e) metals, and (f) chlorinated aromatic compounds and other chemicals of public health concern in industry or the general environment.

Three groups of scientists, totaling approximately 35 individuals, approach these problems from the fields of (1) radiobiology and experimental carcinogenesis, (2) biochemical and environmental toxicology, and (3) respiratory biology and inhalation toxicology. These groups have specific projects ongoing to determine the genetic susceptibility to cancer of human populations, to study how environmental chemicals cause malignant transformations, organ-specific actions of environmental toxins and the biology of pollutant inhalation. In addition, epidemiological studies are in progress to study the effects of air pollutants in ambient and work environments.

Oregon State University

The Oregon State University EHS Center has many of its research programs focussed on the chemical and biological aspects of chlorinated hydrocarbons in

the environment. Major accomplishments have been in those areas that relate to these studies, including chemical identification of environmental contaminants arising from the use of pentachlorophenol and polychlorinated biphenyls. A rainbow trout model system for carcinogenesis has been developed and is being used to determine mechanisms of carcinogenesis, particularly in the liver.

Mount Sinai School of Medicine

The Center at Mount Sinai School of Medicine's major thrust is the clinical and epidemiological study of populations which have been exposed to pollutants, primarily at the work site. Individuals exposed to such pollutants as asbestos, lead, textile dust, printer's ink, and fluoride have been evaluated for possible health effects. Cohorts of exposed persons have been established for future follow-ups, and tissue and fluid samples have been obtained.

Future studies will involve the application to epidemiologic studies of powerful new biologic probes derived from such fields as molecular biology, cytogenetics, and neurotoxicology. The application of these technologies to epidemiologic analyses will permit earlier detection than heretofore of toxic dysfunctions, the identification of larger numbers of affected persons, the detection of dysfunction at lower levels of toxic exposure, and possibly the identification of early manifestations of disease. It is hoped that this process will ultimately permit the earlier diagnosis of toxic diseases and will provide a basis for preventive intervention at relatively early stages when reversal of disease processes may still be possible.

Cincinnati University

The focus of this Center's activity is to better understand a variety of environmental hazards which affect human health in order to be able to prevent and control the detrimental effects. Emphasis is on (1) toxic and essential metals with neurotoxic and nephrotoxic manifestations studied in terms of pharmacokinetics and mechanisms of toxicity at the membrane and molecular levels, (2) pulmonary and inhalation toxicology, including such agents as aerosols, aluminum, fine particles and asbestos, (3) clinical and epidemiological studies in humans involving reproductive, pulmonary, dermatologic and neurologic parameters, and (4) genetic studies to identify and characterize basic mechanism of action of mutagens, carcinogens, and teratogens. Examples of specific studies are:

A major study of environmental mutagenesis and carcinogenesis with specific emphasis on the way the body handles chemicals such as polycyclic aromatic hydrocarbons and metabolizes them to active carcinogens. The Center is also investigating the hypothesis that promoting agents, which appear to be an initial step in carcinogenesis, may act via toxic effects on the immune system.

This Center is also developing a screening system for determining the sensitivity of human airways to irritating pollutants. This would be useful in determining which individuals might be more likely to have excessive reactions to inhaled pollutants in industrial settings. The Center also has a major effort on determining the effects of early lead exposure on the physical and mental development of young children.

New York University

This Center is devoted to the identification, elucidation and control of environmentally-caused diseases. The approach is to study at the molecular level the mechanisms of action of mutagens, carcinogens and other toxicants using pathological, physiological and epidemiological methods and to characterize the transport processes and pathways through which environmental agents reach human tissues. Recent studies include (1) the effects of ozone on the pulmonary function of normal children, (2) carcinogenic studies on benzene in rats and mice, (3) carcinogenicity for nasal mucosa by inhaled direct-acting alkylating agents, (4) radiation therapy and its relationship to breast and thyroid cancer in women, and (5) epidemiological study of AIDS in homosexual men.

In addition, studies have determined that vegetarian populations known to have lower levels of breast, colon, and prostate cancers, might be protected by compounds called protease inhibitors. Synthetic protease inhibitors have been shown at this Center to inhibit tumor promotion, both in experimental animals and in cell cultures.

Massachusetts Institute of Technology

The Center at MIT is studying the complex mixtures emitted from combustion systems (furnaces, diesel motors, etc.), and are determining which pollutants are actually getting into humans in a form capable of reacting with cellular macromolecules. Pollutants are being studied to determine if they are carcinogenic or mutagenic and test procedures are being developed to determine their presence in body fluids.

University of Washington MFB Center

The University of Washington MFB Center provides support for cellular and molecular research using aquatic species models for the study of human disease processes. Principal areas of emphasis include studies of toxic substances such as mutagens, teratogens, and carcinogens. The Center encourages the use of Center facilities by faculty with complementary interests and expertise from the College of Fisheries, Department of Pathology, Bioengineering, Pharmacology, and other University departments, and encourages the development of the use of eggs and embryos of fish and other aquatic species as a tool for the study of genetic and epigenetic toxic injury to the reproductive process and the development of aquatic species models for other diseases when they seem especially promising.

Medical College of Wisconsin MFB Center

This Marine and Freshwater Biomedical Research Center provides core support to investigators using marine and freshwater animal species to elucidate mechanisms of biological response to environmental chemicals which may be applicable to a better understanding of the human response. A variety of approaches are used and include hormonal studies related to obesity, immune mechanisms in rainbow trout which have a comparatively simple immune system, chemical translocation mechanisms under hormonal control, comparative effects of pyrethroid insecticides on excitatory amino acid receptors and comparative corneal physiology to better understand the toxicology of the eye. A particularly important area has

been the use of algae as a model of mammalian metal metabolism. By comparative studies between algae proteins and the corresponding mammalian proteins, these studies are revealing new information on the function, structure, and properties of these proteins, including cadmium-zinc antagonism properties, binding characteristics and the role of zinc as a regulator of cell metabolism.

Duke University MFB Center

The Duke University Marine Biomedical Center promotes and integrates multidisciplinary research on aquatic organisms with a focus on environmental health problems. Ongoing programs of the Center illustrate how marine organisms can be used to an advantage in sorting out the fundamental mechanisms which underlie molecular, cellular and organismic behavior in normal and pathological states. The Administrative Core Unit of the Marine Biomedical Center stimulates research and information exchange concerning problems in the marine sciences that are related to environmental health, coordinates lectures, meetings, and a visiting scholar program.

TRAINING PROGRAM OVERVIEW

Environmental Toxicology

Environmental toxicology can be defined as the study of the adverse effects of environmental chemicals on human health. As such, it is the major training area of the NIEHS. The paragraphs which follow outline our institutional training grants in this area.

University of Michigan, School of Public Health
(Dr. Isadore Bernstein, Program Director)

The program at Michigan focuses on the biochemical mechanisms of toxic action at the cellular and molecular levels and the ability of the organism to neutralize the toxic effects of chemicals or to repair the damage resulting from exposures to such substances. Students are provided with the advanced theoretical and technical instruction necessary for basic investigation of the biological responses to environmental chemicals. Students develop an understanding of man's total chemical environment and applied techniques available for assessing potential chemical hazards to man. The objectives are attained through course work, seminars, laboratory instruction, and participation in research.

University of Kansas, School of Medicine
(Dr. Curtis Klaassen, Program Director)

The program at the University of Kansas emphasizes two facets of environmental toxicology. One is to impart to the students the status of toxicologic problems which are caused by chemicals in our environment and the methods which are used to evaluate these problems. This aspect of training involves didactic material and contact with toxicologists who are engaged in this type of evaluation. General problems such as evaluation of risks for chemicals in food and water, and development of tests for setting standards will be covered throughout the training. In depth, advanced courses are taught in part by toxicologists from industry. The second aspect of environmental toxicology which is emphasized is to learn the general research approaches which are used in answering environmental questions which require the application of scientific research. The focus is on the biological mechanisms involved in the response of the organism to environmental chemicals such as heavy metals, pesticides, radiation, etc. as well as the effect of the organism on the chemical (absorption, distribution, biotransformation, and excretion).

Medical College of Virginia, Department of Pharmacology and Toxicology
(Dr. Albert Munson, Program Director)

Training is provided in a multidisciplinary research environment which is associated with the development of didactic knowledge through formal instructions in biochemistry, physiology, microbiology, pharmacology, toxicology, immunology, and related fields. The core courses in the basic curriculum are biochemistry, physiology, pharmacology, toxicology, and statistics. Students must also complete two advanced courses from biochemical pharmacology, neurochemical pharmacology, advanced toxicology, or behavioral toxicology. Courses offered by the other basic science departments are available and taken by the students to meet specific needs for their areas of research. Rotations are performed through each of the areas during the students' first year. Areas of

research available to the trainees are biochemical toxicology, central nervous system toxicology, analytical toxicology, response surface modeling, and immunotoxicology.

Representative types of research activities are immunosuppression by subchronic exposure to various chemicals, neurobehavioral toxicity of inhaled solvents, alteration of lymphocyte function by environmental agents, enhanced excretion of organochlorine compounds, host-parasite interactions in immunologically altered subjects, herpes simplex virus 2 in host resistance models for immunotoxicology, the effects of phorbol diester tumor promoters on macrophage differentiation and function, Naegleria Fowleri-macrophage interactions, and xenobiotic neonatal imprinting.

The Johns Hopkins University, Department of Environmental Health Sciences
(Dr. Gareth M. Green, Program Director)

The Johns Hopkins Center for Research Training in the Environmental Health Sciences conducts five distinct but coordinated programs with predoctoral and postdoctoral experience in genetic, biochemical, and neurobehavioral toxicology, environmental chemistry, and experimental pathology. Training involves didactic course work, laboratory rotations, seminars, journal clubs, and doctoral and postdoctoral research under the guidance of a faculty preceptor. The educational experience begins with a broad, multidisciplinary core curriculum for all students, a second tier of core curriculum germane to the individual program track, and a set of elective subjects germane to each disciplinary track. The toxicology track emphasizes the quantitative aspects of dose, route of exposure absorption, distribution, metabolism, biochemical effect, and excretion of xenobiotics, and systematic approaches to risk assessment through toxicologic studies. The cell biology and environmental pathology track focuses on the properties of host resistance or susceptibility to chemical agents, biological control mechanisms, particularly those involved in cell differentiation and growth, cellular alterations and correlations of these factors with both acute and chronic pathological responses to environmental agents and conditions. The environmental chemistry track focuses on the molecular basis of toxic chemical effects on biological molecules with a goal toward understanding mechanisms that will allow for predictions of toxicity, risk assessment, and dose extrapolations. The genetic toxicology track is concerned with injury and repair to the genetic apparatus of biological systems caused by chemical and physical agents of the environment. The neurobehavioral program of research training focuses on the enzymatic and cellular basis of neurotoxicity and the nature of behavioral effects of environmental substances.

This multidisciplinary training program combines the diverse multidisciplinary scientific opportunities of a large faculty with disciplinary focus and defined curriculum of the individual training tracks. The overall goal is to graduate competent researchers in focussed disciplines who are knowledgeable and aware of the relationship between their work and broader questions in environmental health sciences.

The University of North Carolina at Chapel Hill, Curriculum in Toxicology
(Dr. Tom S. Miya, Program Director)

The minimum academic core for the Ph.D. degree includes 38 credit hours in didactic courses: biochemistry (biochemical toxicology, and one additional advanced biochemistry course), biostatistics, epidemiology, histology, pathology, pharmacology (principles of pharmacology and toxicology, and advanced toxicology), physiology, toxicology (special problems), and three elective courses. In addition, each predoctoral student participates in 12 credit hours of non-didactic training activities; i.e., two laboratory rotations prior to the doctoral research and four seminars. The completion of appropriate courses during the prior academic career permits a student to have a greater latitude in the selection of courses.

The research interests of the faculty are directed primarily at the biochemical and physiological mechanisms of action in mammalian systems. The interests include most areas of toxicology but major emphases are directed towards biochemical toxicology including xenobiotic metabolism, heavy metal toxicology, neurotoxicology including behavioral toxicology, pulmonary and inhalation toxicology, and carcinogenesis and mutagenesis.

Northwestern University, Department of Pharmacology
(Dr. Toshio Narahashi, Program Director)

The foci of the program are the neurotoxicology of pesticides and heavy metals, degradation of environmental toxicants, and mechanisms of chemical carcinogenesis. The Departments of Pharmacology and Pathology are endowed with strong nuclei of toxicology. Several members in the Department of Pharmacology are actively engaged in the research in the field of environmental toxicology, including the mechanism of action of pesticides and heavy metals on the nervous system, the regulation of cellular metabolism of environmental pollutants and carcinogens such as the polycyclic aromatic hydrocarbon benzo(a)pyrene, the erythrocyte function and energy metabolism as affected by environmental contaminants, the teratogenic effects of methylmercury, the reaction mechanism of liver microsomal cytochrome P-450 in metabolism of xenobiotics, and the mechanism of action of red-tide poisons on the nervous system. Several members in the Department of Pathology are active in research in environmental pathology, including injury of DNA by direct and indirect acting carcinogens in the lymphocyte, carcinogenic action of hypolipidemic agents on rat liver, metabolic activation of chemicals by drug metabolizing enzymes in the pancreas, development of rat chimera for the study of clonal origin of chemically induced preneoplastic and neoplastic lesions, single and repeated infection with influenza virus as a promoting agent in chemically induced bronchogenic carcinoma, identification of promoting agents in urine for chemically induced bladder cancer, biochemical and morphological characteristics of transplantable acinar and ductal pancreatic carcinoma cell lines.

New York University Medical Center, Institute of Environmental Medicine
(Dr. Morton Lippmann, Program Director)

The focus of this program is on inhalation toxicology. Required courses in this program are: environmental contamination, environmental toxicology, introduction to biostatistics and epidemiology, seminar on current problems in environmental

health, experimental methods in environmental toxicology, and special topics in biochemical toxicology. Electives include: environmental physiology of the respiratory system, aerosol science, analytic chemistry of environmental contaminants, environmental hygiene measurements, environmental hygiene laboratory, environmental carcinogenesis, environmental epidemiology, DNA repair and environmental mutagenesis, pharmacology, biochemistry, general pathology, mammalian physiology, and histology.

Research programs available to students are (1) short-term and chronic inhalation exposures to animals in vivo with various endpoints; e.g., carcinogenesis, physiological responses; i.e., airway mechanics and particle clearance, structural changes in lung airways and airspaces, macrophage responses and neurobehavioral responses, (2) short-term inhalation exposures of human volunteers in vivo to airborne irritants with measurement of functional responses, especially particle clearance (3) exposures of lung cells in culture to gaseous pollutants and extracts from particles, and (4) studies of deposition patterns of particles and vapors in hollow airway casts and models of respiratory tract airways.

Oregon State University, Environmental Health Sciences Center
(Dr. Ian J. Tinsley, Program Director)

The objective of this program is to provide predoctoral and postdoctoral training in the area of environmental toxicology with special emphasis directed toward the elucidation of the mechanism of action of environmental contaminants.

Research options for trainees include: (1) biochemical effects, biotransformation and mechanism of action of environmental toxicants (e.g., hexachlorobenzene, pyrrolizidine alkaloids) - comparative studies, (2) mechanisms of chemical carcinogenesis using the rainbow trout model, (3) biomembrane effects - action of PCBs and related compounds and effects of induced lipid alterations, (4) glutathione status and toxicity of environmental chemicals at varying levels of cellular organization - whole animal to purified enzymes, (5) the formation of N-nitrosamines in foods and carcinogenic effect of these compounds in trout, (6) comparative studies of nicotinic receptors at the neuromuscular junction, and (7) metabolism and biochemical effects of substituted fatty acids - brominated, cyclopropene derivatives.

All predoctoral trainees are required to complete 43-44 credits of core courses in biochemistry, physiology, statistics, pathology, toxicology, pharmacokinetics and chemical behavior. Supporting areas include training in analytical techniques both chemical and cellular, biochemical systems (membranes, neurochemistry, nucleic acids, etc.) and the use of aquatic models. A focus of research on the mechanism of toxic action of environmental toxicants is required of both pre- and postdoctoral trainees.

The University of Arizona, College of Pharmacy
(Dr. I. Glenn Sipes, Program Director)

The major focus of the program is understanding mechanisms of toxicity with special emphasis on organ-specific toxicities (liver, lung, kidney). Research activities available for trainees include disposition and biotransformation of

chemicals, toxicity of metals, inhalation toxicology, chemical carcinogenesis, neuroscience, toxicology, and mechanisms of hepatic and renal toxicology. Associated with activities are training in cell and tissue culture, analytical techniques, immunology, chelation chemistry, enzyme purification, etc.

Predoctoral students are trained in the necessary basic science courses (biochemistry, human physiology, and pathology), in advanced toxicology or relevant pharmacology courses (analytical toxicology, biotoxicology, drug metabolism and disposition, and neuropharmacology), and in a number of advanced areas (inhalation toxicology, pharmacokinetics, detection and analysis of environmental materials, environmental toxicology, chemical carcinogenesis, statistics and neurotoxicology). Elective courses allow the trainee to specialize in certain areas. Examples of available electives include environmental carcinogenesis, cancer biology, introduction to pesticides, immunology, molecular and cellular biology, nucleic acids, biochemistry of membranes, advanced instrumental analysis, molecular and cardiovascular-pharmacology, among others. The primary emphasis of the program is on research training by the incorporation of laboratory rotations and research seminars into the curriculum and the requirement that the trainees conduct and defend a dissertation research project.

Trainees are required to present their findings in individual laboratory meetings, formal student research seminars and at regional or national meetings. Both predoctoral and postdoctoral students are encouraged to participate in teaching by presenting lectures in environmental toxicology, nursing pharmacology, clinical toxicology and other appropriate courses. Postdoctoral students with little or no training in toxicology are encouraged to participate in the toxicology courses. All postdoctoral trainees perform original research projects under the direction of a faculty preceptor. They also are expected to prepare small grant applications and to participate in the teaching efforts of the program.

University of California-Davis, Department of Environmental Toxicology
(Dr. Dennis P. H. Hsieh, Program Director)

The environmental toxicology training program consists of a blend of interdisciplinary training accompanied by required coursework and research in environmental toxicology. The core toxicology courses required for students in the program include: principles of environmental toxicology, biological effects of toxicants, legal aspects of environmental toxicology, mammalian toxicology, environmental toxicants, mechanisms of toxic action, analysis of toxicants, analysis of toxicants lab and a seminar. Other elective courses are available from the offerings of related Graduate Groups such as pharmacology and toxicology, agricultural and environmental chemistry, biochemistry, physiology, and pathology.

Research activities are focused in three main areas: (1) toxicology, with emphasis in metabolism and disposition of xenobiotics, mechanisms of toxic action, genotoxicity and carcinogenicity of chemicals, and chemical risk assessment; (2) Environmental fate and decontamination of environmental toxicants, with emphasis on chemical transformation processes and toxic waste disposal techniques, and (3) analytical methods, with emphasis on the

development of new and specific methods designed for trace amounts of individual compounds, their metabolites and decomposition products.

Children's Hospital Research Foundation, University of Cincinnati
(Dr. Ernest F. Zimmerman, Program Director)

This program approaches teratology as the study of abnormal manifestations of development. Study in developmental biology, particularly in the molecular aspects of the subject, is fundamental to the program. Emphasis is placed on the effects of environmental agents that cause molecular perturbations, leading to physiologic morphologic, and functional deficits expressed as congenital malformations.

The course requirements reflect this philosophy. Required core courses are in biochemistry, molecular genetics, developmental biology and teratology. The role of pharmacodynamics, biotransformation of environmental agents and statistics are stressed, with instruction coming from elective courses. In addition to these didactic courses, instruction is supplemented every quarter with journal club, developmental biology seminar, and teratology seminar. To familiarize students with the interests of the faculty, laboratory rotations are required of incoming predoctoral trainees for three quarters. Further, a course in teratology techniques is given to trainees which teaches practical methods of monitoring morphologic defects.

Areas of research include: (1) the role of neurotransmitters in palate development and perturbation of neurotransmitter mechanisms by neuropharmacologic agents to cause cleft palate, (2) mechanisms by which teratogens perturb limb morphogenesis to produce limb defects (alterations in embryonic pH by acetazolamide as a mechanism of teratogenesis is being investigated), (3) teratogen alteration of behavior, studying the anticonvulsant valproic acid, the CNS agent methylazoxymethanol (MAM) an alcohol (Fetal Alcohol Syndrome), (4) potentiative interactions between caffeine and various teratogenic agents, and (5) interactions of teratogens and mutant genes in neural tube defects.

Massachusetts Institute of Technology, Department of Nutrition and Food Science (Dr. Gerald N. Wogan, Program Director)

The training program in environmental toxicology at MIT has as its aim the education and training of persons to conduct independent research on problems of public health importance which concern environmental chemicals and, in addition, to teach others in the same field. The core curriculum of the predoctoral toxicology training program includes the following courses: general biochemistry, analytical practices in biochemistry, cell biology, nucleic acid biochemistry, general toxicology, biochemistry of xenobiotics, genetic toxicology, and two electives. These electives are chosen with the objective of complementing the scope of the educational background of degree candidates beyond the core curriculum. Students are required to act as teaching assistants in at least one course for one term and, in addition, must conceptualize, complete, and defend a thesis based on an original research project.

The focus of the program encompasses a number of areas such as genetic, biochemical, pathological, analytical, and inhalation toxicology. Six full-time faculty members in the program supervise the research activities available to

trainees. These faculty have taken two primary types of research approaches to accomplish the program's objectives: 1) those concerned with understanding mechanisms by which toxic effects are produced, and 2) those intended to improve methodologies through which toxic substances or their effects can be detected and characterized.

University of Mississippi Medical Center
(Dr. Harihara Mehendale, Program Director)

The program at Mississippi is designed to attract graduate students to undertake toxicological research and to prepare them for careers as independent investigators in toxicology. These individuals are given training in modern toxicological and basic medical sciences with emphasis of pharmacological, physiological, pathological and biochemical mechanisms related to the actions of toxic materials. The program is oriented toward the metabolism approach to the evaluation of toxicity of chemicals which may be found in the environment. These chemicals include man-made substances (e.g., food additives, industrial toxicants, agricultural chemicals, air and water pollutants) and naturally occurring substances. Participants are trained in all phases of toxicology including enzymology, isotope techniques, isolation and identification of metabolic products and problems related to the comparative evaluation of toxic effects in various animal species. The program is designed to give training to both predoctoral and postdoctoral students in toxicology. The program involves didactic work, but has its primary emphasis on research activity. In addition, training opportunities are available in some areas of forensic toxicology as well as analytical aspects of clinical toxicology. An integrated core curriculum is implemented which combines courses and seminars in several departments with emphasis on the basic biochemical, pharmacological, physiological, pathological and analytical aspects of toxicology. With this basis, the trainees are directed toward the application of such knowledge and technology for the development of solutions to environmental health problems.

Cornell University, Institute for Comparative and Environmental Toxicology
(Dr. Christopher F. Wilkinson, Program Director)

The environmental toxicology program at Cornell University is a campus-wide program that is administered through the Institute for Comparative and Environmental Toxicology. The main foci of the program are biochemical, nutritional, genetic and veterinary toxicology, ecotoxicology, risk assessment and public policy aspects of toxicology. The program also interfaces with an NIEHS training program in environmental pathology.

The research activities available to trainees include basic studies on the molecular interactions of toxicants with enzymes and genetic material, studies on drug metabolism and on the genetics of cytochrome P-450 and its relationship to immunosuppression, studies on the relationship between diet and cancer, and studies on the fate and disposition of toxicants in the environment and their impact on a variety of species, including man.

Dartmouth Medical School, Department of Pharmacology and Toxicology
(Dr. Roger P. Smith, Program Director)

The core curriculum at Dartmouth includes courses in biochemistry, physiology, pathology, pharmacology and toxicology. A minimum of five electives may be

selected from environment and human health, toxic chemicals and the law, immunology, statistics, pharmacokinetics, general pharmacology, cardiovascular pharmacology, epidemiology, neuropharmacology and a wide variety of graduate and under-graduate courses.

In addition, the program requires three research rotations, proficiency in a foreign language or computer programming, a qualifying examination in the form of a defensible research proposal and a thesis of publishable quality.

The primary focus of the program is training in environmental toxicology. Well funded research programs exist in the areas of pancreatic carcinogenesis, experimental teratology, molecular oncology and biochemical toxicology.

Vanderbilt University, School of Medicine, Department of Biochemistry
(Dr. F. Peter Guengerich, Program Director)

The thrust of the training program at Vanderbilt is in the chemical and biological aspects of molecular toxicology. Predoctoral students in the program fulfill degree requirements in chemistry, biochemistry, or pharmacology in order to best establish a basic research background. The students also take a comprehensive course in toxicology which covers fundamentals of both molecular and applied toxicology. Electives are taken in chemistry, biochemistry, and pharmacology to develop individual students' interest. Other courses in pathology, molecular biology, and biostatistics are also often taken as electives. In general, graduate students begin rotations in individual laboratories during the first year and pick a thesis advisor (and research problem) by the end of the first year in residence.

Some of the major research activities which graduate and postdoctoral students are associated with include the enzymology of proteins involved in metabolic activation and detoxication, studies in the catalytic mechanisms of these enzymes, elucidation of metabolic pathways of biotransformation, characterization and determination of fates of DNA and protein adducts, characterization of tumor antigens and their roles, interaction of metals with chromatin, regulation of the cell cycle, regulation and roles of glutathione metabolism, expression of DNA repair genes, cloning of genes for enzymes of interest in toxicology, eicosanoids and their interactions with toxins, acetaminophen oxidation, characterization of structures, metabolism, and mode of action of toxic natural products, new techniques in mass and NMR spectrometry, synthesis and biosynthesis of toxic natural products, model studies on the metabolism of toxic sulfur compounds, and development of antidotes for heavy metal toxicity.

North Carolina State University, School of Agriculture and Life Sciences
(Dr. Frank E. Guthrie, Program Director)

The primary focus of the program at North Carolina State is on environmental/biochemical toxicology. Pre- and postdoctoral students are required to take a series of courses including graduate biochemistry, biochemical toxicology, pharmacology, epidemiology, pathology for toxicologists, statistics for toxicologists and advised electives including environmental toxicology, methods in toxicology, general toxicology, teratology, pharmacokinetics, special problems in toxicology and seminar. They then elect courses appropriate to their minor from offerings of the program or the large number of courses taught

in associated departments of biochemistry, zoology, physiology, genetics, microbiology, veterinary, science, etc. The preliminary and a final examination are required and the usual length of time is 5 years (B.S. to Ph.D.).

Research training opportunities exist in metabolism, enzymatic pathways, mode of action of toxicants, absorption, distribution, and enzyme purification.

University of Wisconsin-Madison, Environmental Toxicology Center
(Dr. Colin R. Jefcoate, Program Director)

The Environmental Toxicology program at University of Wisconsin-Madison is broadly based, with opportunities for training and research in analysis and testing, pathobiology of chemically-induced diseases, behavioral toxicology and chemicals in the environment. Its interdisciplinary structure allows access to the multitude of resources of the UW-Madison campus as well as offering opportunities for interaction and collaboration among faculty and trainees. The available faculty trainers come from three schools and more than 18 departments.

The core curriculum, required of all environmental toxicology graduate students consists of toxicology, toxicology II, toxicants in the environment (sources, distribution, fate, and effects), and physiological chemistry (or equivalent biochemistry combination). Postdoctoral trainees are expected to audit the toxicology courses and may audit a limited number of additional courses deemed helpful to their research efforts, although the primary emphasis is on their research training. In addition, all graduate students and postdoctoral trainees are expected to attend the weekly environmental toxicology seminar and to enroll and participate in one or more offerings of single topic mini-courses; e.g., toxicology of the dioxins. Additional courses to best serve the graduate student's research interests are selected from the extensive offerings at the UW-Madison. A typical sequence might include: histology, pathology, human physiology and biophysics, experimental surgery and techniques in research animals, introduction to experimental oncology, and viral and chemical carcinogenesis.

University of Rochester, School of Medicine and Dentistry, Division of Toxicology (Dr. Victor G. Laties, Program Director)

The core curriculum at Rochester is grounded in basic courses in biochemistry, physiology and pathology that are taken during the first year in residence. Students are encouraged to take elective seminars during this period as well as to rotate through at least two laboratories before starting their second year and settling on a thesis advisor whose research interests are compatible with theirs. The main toxicology course is taken by students during their second year along with a basic course in pharmacology. These courses are meant to bring the students up-to-date on the current status of research in a wide variety of areas, and give them a thorough grounding in the basic principles of the effects of toxicants. Specialty seminars supplement the core curriculum and include topics in developmental toxicology, behavioral pharmacology and toxicology, evaluation and control of air pollution, aerosols, pulmonary toxicology, in vitro methods, and immunotoxicology. Students are encouraged to participate in seminars during each semester of their graduate career.

The four main areas of emphasis for research training in this program are neuro-behavioral toxicology, inhalation toxicology, developmental, reproductive and genetic toxicology, and cellular and molecular toxicology.

Environmental Epidemiology

Environmental epidemiology differs from clinical epidemiology in that the investigation is not the determination of the source of a communicable disease. Rather, environmental epidemiologists study situations in which there are known exposures of human populations to environmental agents and determine if these exposures result in untoward health effects. The following programs train researchers for careers in environmental epidemiology and/or biostatistics.

UCLA School of Public Health
(Dr. David Wegman, Program Director)

A collaborative training program at UCLA involves the Divisions of Epidemiology, Biostatistics, and Environmental Sciences and trains individuals through courses relevant to environmental health, epidemiology, and biostatistics. Curricula are organized according to whether the individual trainee wishes to emphasize training in environmental and occupational health sciences, epidemiology, or biostatistics. Core courses required of all trainees include human disease in public health, principles of epidemiology, introduction to biostatistics, and environmental and occupational health. Trainees may then select from a variety of advanced didactics and research programs toward degree and/or postdoctoral studies.

Yale University
(Dr. Jan Stolwijk, Program Director)

The training program at Yale provides research training in environmental epidemiology and environmental biostatistics. The program leads to a Ph.D. or Dr.P.H. in epidemiology or biostatistics. There is provision for the support of individuals with prior doctoral degrees who are candidates for additional degrees. Graduate study consists of two years of course work in environmental health sciences, chronic disease epidemiology, and biostatistics. Trainees then complete a comprehensive examination at the end of the course work followed by original research and preparation of a dissertation.

Harvard University, School of Public Health (Dr. Richard R. Monson and Dr. James H. Ware, Program Directors)

The epidemiology program consists of research training in environmental and/or occupational epidemiology. All students take intermediate courses in epidemiology, biostatistics and environmental health. All students take advanced courses in epidemiology. Depending on training interest, students also take courses in toxicology, occupational health, industrial hygiene, cancer, biology and physiology.

Examples of research activities include follow-up studies of women treated with radiation for benign gynecologic disease, relation between birth defects and occupational exposures (rubber industry, Viet Nam service), environmental factors associated with abnormal outcomes of pregnancy, neurotoxic effects of solvent exposure, respiratory effects of exposure to beryllium, adverse effects of exposure to therapeutic drugs, adverse effects of exposure to lead, relation between environmental exposure to selenium and myocardial infarction, and effects of occupational exposure to machinery fluids.

In the biostatistics program, students in the doctoral program are required to take two years of coursework, and most complete the equivalent of three years of classroom work. The core curriculum includes a one-year sequence in probability and mathematical statistics, and courses in linear models, discrete multivariate analysis, and biometry, the latter emphasizing survival analysis. Students also take advanced courses in multivariate methods, data analysis, stochastic processes, statistical computing, and decision theory. Students may take courses offered at other Harvard Schools and at MIT under a consortium agreement. In January of the second year, doctoral students take a written qualifying exam. Upon passing this examination, students begin to develop a dissertation topic in collaboration with a faculty advisor. When the research plan has been formulated, the doctoral student presents the proposal to an examining committee, which also examines the student in major and minor fields. After completing this oral examination, the student begins the proposed program of doctoral research.

Students in the environmental health sciences training program participate in the Department's special seminars and working groups on statistical methods for environmental health and choose dissertation topics suggested by the Department's environmental research program. Current research interests in the Department include methods for the assessment of exposure to environmental hazards, design and analysis of the animal carcinogen bioassay, methods for longitudinal studies in non-experimental settings, and problems in combining evidence from several sources. Emerging research issues include acid rain and assessment of the health effects of water-borne chemicals and toxic waste sites. In each of these areas of application, the emphasis is on innovative statistical research to develop improved methods for the design, conduct, and analysis of investigations.

University of North Carolina-Chapel Hill, School of Public Health,
Department of Biostatistics (Dr. Lawrence L. Kupper, Program Director)

This training program is designed to train biostatisticians at the predoctoral and postdoctoral levels to conduct biostatistical research relevant to important environmental health problems and to provide high-level statistical consulting support to other research workers in the environmental health field. The academic courses include all those in mathematical and experimental statistics which are the core of a doctoral degree program for a professional statistician working in the health sciences area, plus supporting program courses concerned specifically with the nature and problems of the physical environment and its biological effects. Training in consultation and research generally involves interaction with workers at the National Institute of Environmental Health Sciences and at the Environmental Protection Agency; these agencies encourage active collaboration with trainees on environmental research problems of mutual interest.

The research activities with which trainees are involved include the design and analysis of toxicological experiments concerning teratogenesis, mutagenesis, and carcinogenesis, the design and analysis of environmental and occupational epidemiological studies, and risk assessment.

Environmental Pathology

Environmental pathology is the study of the effects of environmental agents on mammalian tissues at the functional gross and histopathological levels. The following institutions have NIEHS support for training environmental pathologists.

Washington University (St. Louis) School of Medicine
(Dr. Charles Kuhn, Program Director)

The environmental pathology training program at Washington University is designed to train pre- and postdoctoral students to apply the approaches of experimental pathology to the investigation of environmental influences on health and disease. The program emphasizes understanding the mechanisms by which environmental factors influence health. Trainees get an overview of the major issues in environmental pathobiology and a detailed understanding of one particular problem area. General knowledge of environmental health problems are developed through a combination of lectures, seminars, and formal courses and research skills are developed through participation in laboratory research aiming at imparting a rigorous empirical approach to analyzing the role of environmental agents in human disease. Trainees can select among the following areas: chemical carcinogenesis, environmental pulmonary disease, effects of environmental agents on the adult and developing nervous system, radiation biology, interactions of environmental agents with the immune system, and basic mechanisms of cell injury and cell death induced by environmental agents. Investigative approaches include biochemistry, cell biology, morphology, and immunology.

State University of New York at Stony Brook
(Dr. Aaron Janoff, Program Director)

The training program at SUNY Medical School trains scientists to study the environmental health effects of inhaled substances on the structure and function of molecules, cells, and tissues of the respiratory system. Participating departments are pathology, medicine, biochemistry, and pharmacology. Research topics include the effects of pollutants on lung enzymes, patterns of clearance of particles from alveoli and their mechanisms, effects of energy related pollutants on the respiratory functions and pulmonary defense mechanisms, adverse effects of drugs on metabolism and morphology in organ and tissue culture, health effects of atmospheric pollutants resulting from energy generation and utilization, pathophysiology of thrombus formation and resolution, repair as a result of exposure of cells to physical and chemical agents, effects of toxic agents on the properties of phospholipid bilayer membranes, and membrane-bound enzymes, and development of sensitive and specific non-invasive techniques for early detection of pulmonary disease.

Duke University Medical Center
(Dr. William Lynn, Program Director)

The training program at Duke has a primary emphasis on pulmonary problems encompassing a wide spectrum of toxicological research. Areas of training come from a variety of departments, including: anatomy, biochemistry, chemistry, environmental studies, marine sciences, microbiology, immunology, pathology,

pharmacology, physiology, and zoology. Courses from these departments are intergrated into a program to produce scientists with the skills and abilities to advance toxicological research and to make appropriate safety assessments of hazardous environmental chemicals.

Colorado State University, College of Veterinary Medicine and Biomedical Sciences, Department of Pathology (Dr. Stephen A. Benjamin, Program Director)

The goal of this program is to produce investigative veterinary pathologists with a research background in environmental pathology. This is a postdoctoral program for DVMs with prior pathology training. It is designed to enable trainees to enter research careers concentrating on the biologic effects of toxic agents in our environment. This is a collaborative program between the Department of Pathology, Colorado State University (CSU), Fort Collins, Colorado and the Inhalation Toxicology Research Institute (ITRI), Lovelace Biomedical and Environmental Research Institute, Albuquerque, New Mexico. The basic training for all participants is at CSU and where trainees are enrolled as candidates for the Ph.D. degree. The core curriculum includes courses in pathology, general toxicology, biostatistics (at CSU) and multidisciplinary inhalation toxicology (at ITRI). Trainees also participate in practical pathology experience and take elective courses honed to their research interests.

Trainees have the opportunity to conduct their toxicologic pathology research at either CSU or ITRI. Research at CSU includes developmental immunotoxicology, naturally recurring phytotoxins, neurotoxicity caused by agents which impair energy metabolism, and chronic oxalate toxicity. Research at ITRI is concentrated on inhalation toxicology involving dose/response relationships of several chemicals and radionuclides, factors which modify biological responses to toxic agents, and the effects of inhaled toxic agents on pulmonary defenses.

University of Washington, School of Medicine, Department of Pathology (Dr. N. Karle Mottet, Program Director)

The objective of the training program at UW is to produce environmental research scientists whose background is based in toxicology and cell/molecular pathology and will function as principal investigators in future research grants. A core faculty of eighteen offers unusual opportunities for training in toxicology and pathology on the cell and molecular level particularly as they relate to alterations of development mechanisms such as to metabolism of the agents, biotransformation and their effects on the evolving cell structure and function. Although trace metals and polycyclic aromatic hydrocarbons are the principal agents studied, others are also subject for investigation. In addition to the usual laboratory animals, the program has excellent facilities for the use of primates and aquatic embryos for research. The Infant Primate Research Lab is a leading one of its kind in the world and provides a unique opportunity to study developmental effects of toxic chemicals on morphologic and behavioral development. Predoctoral trainees take a core of environmental toxicology and pathology (and related prerequisites) including a three quarter course in cell and molecular biology of diseases. Each person's program is designed individually, and thesis research project must be relevant to the environmental sciences. All predoctorals are assigned a faculty advisor from the time they enter the program. Their supervisory committee must include a member of the advisory committee of this training program. During their first year they are

required to take a laboratory rotation into three laboratories of dissimilar methodology (morphology, biochemistry, cell culture, etc.) before making final selection of a permanent laboratory during the second year. At the end of the second year of training, a student is required to take a "General Exam" and, for those who pass, devote his/her principal efforts in research during the third to fifth year followed by thesis defenses in a final examination. Completion of a Ph.D. usually takes five years. Postdoctoral trainees are selected by the advisory committee in consultation with the laboratory director in which the person seeks to work. Trainees are expected to fill in any deficits in his/her training as pertains to the above core courses and through auditing courses and seminars add a new dimension to their expertise. All pre-and postdoctoral trainees present their research annually in a trainee seminar. Graduates are trained solely for academic careers as principal investigators to design and carry out independent research.

Michigan State University, College of Veterinary Medicine
(Dr. Robert W. Leader, Program Director)

The program at Michigan State is designed to train veterinarians and physicians in a combination of pathology and toxicology with the goal that the persons who finish this program will be able to lead research efforts in fundamental toxicology but with the clinical perception and training gained from their experience in pathology. There are three phases in the training: 1) each candidate is required to finish the graduate studies core curriculum in pathology which includes classroom studies, laboratories and rotations in autopsy and surgical pathology, 2) this is followed by a five week practicum rotation in each of three toxicology research laboratories to gain a broad view of the field of toxicology, and 3) trainees then enter a research program in a funded toxicology laboratory to complete their Ph.D. thesis research. All trainees are required also to present seminars both on campus and at professional meetings.

The focus of the program is to achieve training in a combination of pathology and toxicology. Twelve laboratories, all funded by outside agencies to do research in toxicology, have consented to supervise the research training of these candidates once they have finished their pathology background. Projects available for these individuals include basic research on the mechanisms of toxic substances in the Pathology/Toxicology Department where there are programs on renal, respiratory, and other types of toxicology, in addition to carcinogenesis studies. Also, the Carcinogenesis Laboratory, a basic research unit in cellular aspects of carcinogenesis has an extensive and well-funded program. Some candidates are trained in the Biochemistry Department where the major thrusts are in the areas of superoxide metabolism, the cellular effects of toxins such as polybrominated biphenyls and other halogenated hydrocarbons. Other possible training sites include the Pesticide Research Laboratory which does fundamental metabolic research on such materials as the dioxins and related materials, the Department of Pediatrics which conducts research on the cellular mechanisms of promotion and initiation in carcinogenesis, and the Department of Pathology which has a program in the pathogenesis of lesions caused by toxic substances including those which act as promoters and initiators.

The University of Vermont School of Medicine, Department of Pathology
(Dr. John E. Craighead, Program Director)

The core courses in this program include biochemistry, histology, cytology, cell biology, seminar, basic pathobiology, toxicology, statistics, and immunology. Students have the opportunity to choose elective courses in areas of particular interest. Students are also exposed to autopsy pathology and cytogenetics. Most students have the opportunity to conduct electron microscopy and tissue culture as part of their research.

Postdoctoral trainees work principally in the laboratory of their chosen preceptors on basic research problems. Courses are available to those who wish to increase the diversity of their formal background, but course work is not required except that postdoctoral trainees are expected to complete an advanced course in basic pharmacology. Customarily, the postdoctoral training period is two years. All trainees participate in a journal club and the pathology research conference. Other basic science departments and the cell biology program also offer weekly seminars.

There is a wide diversity of research programs which are broadly considered environmental pathology at the University of Vermont College of Medicine. These include: a) asbestos carcinogenesis, b) pathogenesis of mineral lung disease in man and experimental animals, c) association of metal accumulation in the CNS in human dementia, d) chemical toxicity and viral susceptibility of beta cells of the islets of langerhans, e) mechanisms of mucin secretion in the respiratory tract, f) genetic toxicology, g) fundamental molecular biology of DNA, and h) fundamental mechanisms of pulmonary fibrosis and bronchiolar disease of lungs.

University of California-Davis, School of Veterinary Medicine
(Dr. Donald Dungworth, Program Director)

The program at UC-Davis uses the format of a specially-tailored Ph.d. degree in comparative pathology and is designed to take three years, assuming trainees have one year of residency training in pathology prior to entering the program. The first 12-18 months of training are spent mainly in taking advanced coursework and in becoming familiar with environmental research programs on the campus. There is continued exposure to hospital pathology by means of conferences and rounds. Required courses are advanced special pathology, environmental toxicology, tumor biology, ultramicroscopic anatomy, medical statistics, and immunology. Elective courses can be in teratology, inhalation toxicology, radiation biology and various biochemistry/molecular biology offerings. The last 18-20 months of the program are spent principally on a research project for the Ph.d. degree within one of the environmentally-related research units.

Major research projects available for potential thesis topics are (1) inhalation toxicology -- effects of photochemical air pollutants and pollutants arising from nuclear or coal-burning power plants, (2) teratology -- teratogenic effects of drugs and chemicals in monkeys, (3) environmental toxicology -- effects of pesticides and natural carcinogens such as aflatoxin, and (4) radiation toxicology -- effects of x and gamma radiation.

Cornell University, College of Veterinary Medicine, Department of Pathology
(Dr. Robert M. Lewis, Program Director)

The purpose of the toxicological pathology training program is to provide veterinary postdoctoral training in environmental pathology which emphasizes the principles and practices of toxicologic pathology. Prerequisites include at least one year of internship training in veterinary pathology, demonstrated research potential, admission to the graduate school of Cornell University and requires active participation in each of the following components: (1) applied pathology, through active participation in departmental necropsy and surgical pathology services, for a minimum of six months; (2) graduate course work to include the following core courses - mechanisms of disease, principles of toxicologic pathology, introductory chemical toxicology, molecular toxicology, safety evaluation in public health and the following elective courses, physiologic disposition of drugs and chemicals, genetic toxicology, diagnostic toxicology, and clinical toxicology.

A research laboratory rotation includes short-term training (up to three months) in a toxicology/pharmacology/pathology research laboratory to allow participation and development of working knowledge of techniques employed by investigative personnel in that environment and an externship of one month of visitation and participation in any of the following research laboratories or a mutually acceptable research environment: Carcinogenesis Testing and Research Laboratories, NCI, Hazelton Laboratories, or Bristol Meyers, Inc.

The types of research activities available for trainees include, as noted earlier, the practicum or short-term toxicity study, the research laboratory rotation (on-campus experience), the externship (off-campus experience), and the student's involvement in a selected thesis research project.

The University of North Carolina School of Medicine, Department of Pathology (Dr. Joseph W. Grisham, Program Director)

Trainees pursuing the Ph.d. degree generally take a core curriculum which includes courses in general pathology, computer science, statistics, and methods courses on necropsy techniques, use of animals in research, and laboratory methods in experimental pathology. Popular elective courses taken by many students include seminar on carcinogenesis, nucleic acid biochemistry, biochemical toxicology, cell biology, DNA replication and mutagenesis. The aim of the formal courses is to provide solid background in the cellular and molecular biology of diseases and the identification of disease-causing agents.

The program has two major areas of research. One concerns chemically-induced toxicity, mutagenesis and carcinogenesis; the other concerns heavy metals toxicity, emphasizing neurotoxicity. In both areas, training focuses on investigation of pathogenesis and basic mechanisms of environmentally-induced disease. Research areas that have been pursued by trainees include: studies of variations in susceptibility to toxicity, mutagenesis, and carcinogenesis as functions of the cell cycle; studies using antibodies to localize and quantitate environmental chemicals; studies comparing the cell biology and responses to environmental chemicals between species for specific target tissues; and studies investigating the cell toxicity induced by organometal compounds. Many studies focus on the use of human cells and tissues. Research skills learned by

trainees span a broad spectrum of modern laboratory techniques. Trainees utilize electron microscopy with elemental analysis and morphometry, various techniques of cell and organ culture, antibody production and quantitation, high pressure liquid chromatographic separations of metabolites and products, and other methods of modern molecular biology, including nucleic acid purification and labeling, various blotting techniques and gene transfection. Most trainees also get some exposure to the experience with conventional histopathology and histochemistry.

Environmental Mutagenesis

Environmental mutagenesis is the study of the interaction of environmental chemicals and other factors on genetic material to determine if potential exists for carcinogenesis or the development of heritable mutations. This is the smallest area of support by the NIEHS.

University of California-San Francisco, Laboratory of Radiobiology and Environmental Health (Dr. Zena Werb, Program Director)

The major part of the training program at the Laboratory of Radiobiology and Environmental Health involves individual research projects under the supervision of faculty preceptors. In addition, the laboratory offers a series of short courses in which basic biology pertinent to the study of environmental mutagenesis and carcinogenesis is covered; e.g., a one-quarter course on DNA repair, a short course on embryonic chimeras and one on radiobiology. All of the trainees attended seminars within the Laboratory of Radiobiology as well as innumerable seminars in other departments, and have all been scheduled to deliver seminars themselves. The trainees also participate in the weekly journal clubs and in informal weekly research meetings.

The focus of the program is on basic research, especially as it applies to environmental mutagenesis and carcinogenesis. The types of research activities available for trainees include: carcinogen-DNA interactions, DNA repair and replication in human genetic disorders, molecular biology of eukaryotic DNA repair genes, DNA replication and repair in normal and abnormal mammalian cells, cell differentiation, cell lineages and commitment in mammalian embryos, quantitation of radiation and chemical damage and repair in germ cells and embryos, cell and molecular biology of extracellular matrix synthesis and degradation, endocytosis and secretion by macrophages, cytogenetics (chromosome aberrations, SCEs), environmental mutagenesis, radiation genetics, vascular repair including purification and regulation of angiogenesis factor from macrophages and studies on regulation of capillary endothelial cell biology including locomotion.

The University of California, Berkeley
(Dr. Stuart Linn, Program Director)

The program at Berkeley is aimed at providing training in environmental mutagenesis and carcinogenesis. The curriculum includes laboratory and classroom instruction in the biochemistry of cultured animal cells, mutagenesis, information transfer and gene expression, biological regulation, nucleic chemistry and biochemistry, etc. Research is conducted in the areas of carcinogenesis, tumor, biology, mutagenesis, and mechanism of DNA repair.

Case Western Reserve School of Medicine
(Dr. Helen Evans, Program Director)

The purpose of the program at Case Western is to train students in the field of genetic toxicology with special emphasis on investigations of the mechanisms of mutagenesis and carcinogenesis. The program is molecularly oriented in its

emphasis of biochemistry, microbiology, and pharmacology. The mechanisms of mutagenesis and carcinogenesis are emphasized. There is a core curriculum in cellular and molecular biology followed by independent research under the supervision of an established investigator.

Research Highlights

Research Grants Program

The research supported through the Research Grants Program is divided into four program areas: (1) characterization of environmental health hazards; (2) biological response to environmental health hazards; (3) applied toxicological research and testing; and (4) biometry and risk estimation. The mechanisms of support in this program include regular Research Grants (R01), Program Project Grants (P01), Conference Grants (R13), New Investigator Research Grants (R23), Small Business Innovation Research Grants and Contracts (R43/N43), Research Career Development Awards (K04), and Minority Biomedical Research Grants (S06).

The following are some research highlights representing four specific areas of investigation: (1) Air Pollution, (2) Electromagnetic, (3) Chemical Interactions and (4) Immunotoxicology.

Air Pollution

Through inhalation of mineral particles, chemical vapors and gases, and polluted air, the respiratory system is frequently exposed to noxious or toxic stimuli. The response of the respiratory system to these challenges is complex, and at present poorly understood. The NIEHS is supporting a variety of studies to determine both the biological effects of exposure to noxious agents and the mechanisms of the toxic response.

Exposure to mineral dusts can induce a number of different responses by the lungs depending on the particle type, the status of the bodies defensive mechanisms and the presence of other environmental exposures. Silica and asbestos exposure have both been shown to be toxic to the lungs, however, the mechanisms for the toxicity has yet to be determined. A model system using the macrophage cell line has been developed to determine the mechanisms of their toxicity. Using this model, it was shown that exposure to either silica or asbestos fibers resulted in the accumulation of calcium accompanied by a loss of cell viability. However, this irreversible cell injury was found not to be caused by the depletion of ATP, but due to some other toxic reaction(s). In other studies, acute asbestos toxicity to pulmonary parenchymal cells was found to be due to both direct effects on the cells as well as indirect actions triggered by the release of oxidants from macrophages during attempted phagocytosis of long asbestos fibers. Based on these and other data, a possible mechanism of irreversible cell injury resulting from asbestos exposure has been developed. In this hypothesis, the interaction of the macrophage with the asbestos particles is thought to be the initiating event which leads to a series of biochemical reactions ultimately resulting in cell death. The specific features of this hypothesis are currently being tested.

Exposure of the respiratory system to noxious chemicals is of concern for both the industrial and public settings. Pyrrolizidine alkaloids such as monocrotaline (MCT) are produced by plants which intoxicate humans and livestock in the U.S. and other countries. MCT produces lung injury accompanied by pulmonary hypertension and right ventricular hypertrophy in experimental animals. Monocrotaline pyrrole (MCTP), a reactive pyrrole metabolite, causes

endothelial cell damage, pulmonary hypertension, and right ventricular hypertrophy in the rat by an undetermined mechanism. This study has examined the role of serotonin (5HT) derived from the platelets in the cardiopulmonary response to MCTP. To investigate the role of 5HT, the effect of two 5HT receptor antagonists was examined in MCTP-treated rats. Neither metergoline nor ketanserin substantially affected the cardiopulmonary response of MCTP-treated rats. These results suggest that interaction of 5HT with its receptors is not involved in the cardiopulmonary response to MCTP. Studies have been initiated to investigate the possibility that prostaglandins may be involved in MCTP toxicity.

The combustion of fossil fuels produces aerosols in the ambient air of most cities. Epidemiological studies associate the exposure of man to air pollution, especially urban aerosols, with excess deaths, exacerbation of chronic lung diseases, and increased incidence of chronic lung diseases. The compounds in the urban aerosol responsible for these effects and the mechanisms by which these effects are brought about are unknown. However, the interaction of heavy metals and polycyclic aromatic hydrocarbons in the atmosphere may be important in causing some of these effects. In order to define the causative role of this interaction, rats are being exposed to aerosols of heavy metals using a newly developed head only exposure model. This model was shown to be a reproducible method for exposing rats to soluble aerosols. Repeated exposures for 4 hours per day, 5 days per week for up to 60 days are possible. This apparatus is currently being used to examine the effects of inhaled NiCl_2 . Rats were exposed to 100, 200, 400, 600, or 1,000 $\mu\text{g}/\text{m}^3$ for 2 hours. The half-life of Ni in the lung was found to be correlated with the lung burden, being 24.8, 25.7, 30.1, 38.5, or 49 hours, for the above exposures, respectively. The kinetics for removal followed the integrated form of the Michaelis-Menton equation. These and other kinetic analysis data illustrate that the toxicokinetic models developed, can reliably predict the lung burden of repeated chronic exposures as would occur in urban polluted air. The utility of this approach in predicting human lung burdens from urban or cigarette sources of nickel aerosols may be possible with this model.

Another source of respiratory injury is exposure to acids or oxidants which may be present in polluted air. These types of studies are taking on increased significance in light of reports of deleterious effects of "acid fog" on human health. The studies supported in this area examine a number of biological endpoints, but the ultimate aim is to determine the health effects and mechanisms of action of atmospheric pollutants.

In one study, the cytotoxic effects of naphthalene derivatives in the mouse lung were examined. Mice were exposed to naphthalene (N), 2-methylnaphthalene (2-MN), and 1-methylnaphthalene via inhalation of vapors. Exposures were for 4 hours at 2 dose levels, and an estimate of the absorbed dose was made based on the assumptions regarding minute ventilation of the mice. The exposure levels for all three compounds were approximately 4.8 mmol/kg and 6.4 mmol/kg. Lung damage included necrosis of the Clara cells which is a nonciliated lung cell, and the damage was restricted to the bronchiolar epithelium. Studies are now being done to determine if upper respiratory damage also occurs after exposure to these vapors.

A series of studies have been initiated to investigate the biochemical mechanisms governing ozone-induced changes in pulmonary functions and bronchial reactivity. Airway permeability was studied in guinea pigs at 2, 8, and 24 hours after a 1 hour exposure to either filtered air or 1 ppm ozone. The level of ozone exposure caused a significant increase in horseradish peroxidase activity in plasma at 2 and 8 hours post ozone exposure and a recovery at 24 hours post exposure. The role of histamine in this reaction was investigated. It was noted that histamine amplified the ozone-induced increase in horseradish peroxidase activity in the plasma. This histamine potentiation of ozone-induced permeability changes may itself be another manifestation of ozone-induced hyperactivity. Pulmonary function studies on guinea pigs exposed for 1 hour to 1 ppm ozone have demonstrated that some long lasting effects occur as the result of this short-term exposure. Lung volumes are decreased in ozone exposed guinea pigs. In general, volumes were decreased maximally to 75 percent of control at 8 - 24 hours post exposure and began to return to control thereafter. These and other volume change observations suggest that airway constriction after ozone is prolonged with some parameters still below control at 48 hours post exposure. The determination of the biochemical parameters behind these permeability, volume and functional changes is the next goal of this research endeavor.

The ability of the respiratory system to remove foreign materials can be affected by environmental pollutants. One project is underway to provide information on the role of NO_2 and O_3 in producing dysfunction in lung defense, and the relationship² between effects and the pathogenesis of lung disease. A group of rabbits underwent control mucociliary and alveolar clearance tests prior to introduction into a test series involving acute exposure to ozone. Four groups of 5 animals each were then exposed for 2 hours to 0.0 (sham), 0.1, 0.6 or 1.2 ppm O_3 followed by tracer aerosol inhalation. All of the O_3 exposures produced a transient slowing in mucociliary clearance rate. The lowest level of ozone (0.1 ppm) produced an acceleration in alveolar clearance, while the higher ozone levels resulted in relatively slower long-term clearance rates. Groups of 5 rabbits underwent 2-hour exposures to NO_2 at 0 (sham), 0.3, 1.0, 10 and 20 ppm followed by tracer aerosol inhalation. There was no significant change in mucociliary clearance rate at any of the concentrations used. For alveolar clearance an apparent dose related response was evident, indicating enhanced clearance at the lowest NO_2 level, with a slowing of clearance towards the control values as the NO_2 concentration increased. The overall clearance findings suggest that the observed changes in alveolar clearance, were not due to dysfunction in tracheobronchial clearance since no changes were seen in the latter. Another group of rabbits was exposed for 2 hours to 1 ppm NO_2 , with sacrifice of 5 animals at 0, 1 day and 7 days post exposure. Bronchopulmonary lavage was performed, followed by analysis of macrophage function. There were no significant differences in total cell number, viability or macrophage number between the three time points. An aliquot of macrophages, in suspension, was then incubated with latex microspheres (3.0 μm) for 15, 30, 60 and 90 minutes. Slide presentations of cells were prepared and the percentage of macrophages that were phagocytic, i.e., ingesting ≥ 1 particle, and the mean number of ingested particles per macrophage were determined. A depression in phagocytosis was observed for the 90-minute incubation period in macrophages immediately recovered from animals exposed to 1.0 ppm NO_2 , when compared to

those of sham or unexposed rabbits. This depression was not seen at 1 or 7 days post exposure.

One project is oriented on experimental immunochemical studies, supplemented by theoretical analyses of antigenicity and structure, of the role of metal-binding proteins (BP), and particularly metallothionein (MT), in metal-associated toxicities. In the past year, a series of collaborative analyses with investigators in this country and abroad (England, Wales, Scotland, Norway, Spain, Switzerland) have been made, primarily by radioimmunoassay (RIA), of MT content in various tissues, cytosols and physiologic fluids of humans and laboratory animals exposed to toxic metals such as cadmium (Cd), gold (Au) and platinum (Pt). A preliminary study (continuing) found that lung tissue from rats exposed to airborne Cd exhibited a sharp rise in MT content after a few exposures, a response paralleled in alveolar macrophages but not observed in polymorphonuclear cells. A study was completed involving the role of MT in the zinc deficiency characteristic of spina bifida; the association between MT and the disease was demonstrated to be tenuous.

These wide-ranging studies mentioned above reflect the multidisciplinary approach being taken in order to define and understand the influence of environmental factors on the respiratory system.

Electromagnetic

The increasing exposure of humans to radiofrequency and microwave electromagnetic fields has provided an impetus for studies evaluating possible bioeffects. Some of the studies on the effects/interactions of electromagnetic energy on biological systems being supported are described in the following paragraphs.

A realistic three-dimensional model for the deposition of electromagnetic energy in man and rats is being developed. This model is based on a newly developed impedance method where the biological body or a part thereof may be represented by a network of impedances whose values are obtained from the complex conductivities of the various regions of the body. When verified, this model will be useful for dose determinations for many of the most important environmental situations for radiofrequency exposure such as broadcast stations and biomedical applications for hyperthermia.

The effects of microwave radiation on the brain is being investigated due to its sensitivity during development and its inability to recover from most injuries. Rats irradiated for 24 hours, 5 mW/cm² on gestational day 16 or 20 exhibited no alteration in cortical thickness or cortical layer development. In another study, exposure of rats for 10 minutes or 1 hour to 25 mW/cm² or 50 mW/cm² (thermogenic levels) of 2.45 GHz microwave radiation elicited endocrine responses similar to other stressors. Increased corticosterone and prolactin₂ and decreased thyrotropin were observed following 1 hour exposure to 50 mW/cm² radiation.

Biopsychological studies of microwave irradiation effects are also underway. These studies have revealed that the rat, whether awake or under anesthesia,

can exhibit marked differences in body temperature during microwave irradiation. The classically defined "core" temperature, as indexed by colonic measurement, may undervalue the temperature in vital brain-stem structures by as much as 3°C. The discrepancy resolves the interpretive difficulty of an earlier set of findings in which the changes in colonic temperature induced by high power microwave exposure were not correlated with changes in velocities of the visually-evoked electrocortical potentials (VEP). With conventional heating there is a close correlation between colonic temperature and VEPs. It was found that in the conventionally warmed animal the core and brain-stem temperatures are similar, but can differ markedly when the animal is warmed by microwaves. These findings have import for clinical, non-localized exposure of human patients and for adventitious overexposure of industrial and military personnel to deeply penetrating, microwave and shortwave fields because a non-excessive elevation in rectal or oral temperature may fail to index an excessive elevation in brain temperature, and may thereby preclude measures to avert or minimize brain damage.

Studies are also being done on the effects of weak electromagnetic fields on single cell organisms. Exposure of Amoebae to weak electromagnetic radiation of 25, 10 and 5 Hz for 48 hours resulted in elevated ATP levels of about 8 percent and the cell surface profiles were altered. These changes were independent of the repetition frequency. No effects on oxygen consumption were noted. Preliminary analysis of the data indicates these effects are caused by the magnetic field component of the radiation.

Chemical Interactions

Since man is exposed to a complex chemical environment, it is essential that knowledge is developed regarding the biological effects of chemical interactions which may result in additive, inhibitory or synergistic actions. The need to study chemical interactions as they relate to biologic effects is only beginning to be appreciated and the scientific basis for evaluating these effects are primitive indeed. Studies on chemical interactions include biotransformations that lead to interactive effects as well as dose response, absorption, and distribution of chemicals within the body. Such basic research should provide additional understanding of interspecies extrapolation necessary to relate animal studies to the human situation.

The NIEHS Extramural Program reports approximately six million dollars in support of various aspects of chemical interactions. The following summarizes a number of these studies and illustrates a variety of approaches to the problem.

Various environmental and pharmaceutical agents may exert biological toxicity by redox-cycling in aerobic cells to generate fluxes of oxygen and/or organic free-radical species. These radicals may interact with transition metals to generate the hydroxy radical, the most oxidizing species known. Paraquat and adriamycin are examples of redox-active agents which generate free radicals. One aspect of this study using paraquat to generate radicals has been to develop a system suitable for the in vitro study of damage to purified enzymes. This will enable investigation into the roles of specific radical species and specific scavenger and protective systems for damage which has previously been pinpointed to specific enzymes.

One study is designed to test the hypothesis that exposure to polyhalogenated aromatic hydrocarbons may render an individual hypersusceptible to renal damage by nephrotoxics through alteration of renal biochemical pathways responsible for the metabolism of a variety of nephrotoxics. Mouse studies have shown that renal cortical slices preincubated with CHCl_3 either in the presence of carbon monoxide or at 0°C , diminished or blocked toxicity, whereas pretreatment with diethyl maleate to reduce protective glutathione concentrations resulted in enhanced nephrotoxicity of CHCl_3 . These experiments demonstrate that the kidney may metabolize CHCl_3 in situ to a nephrotoxic metabolite.

Metals are ubiquitous environmental contaminants and their interactions with other contaminating agents is an important area for investigation. Metal interactions with known mutagenic agents using short term (bacterial) assays for mutagenicity are being examined. Metals of interest include chromium, cadmium, arsenic, beryllium, lead and mercury. Some findings are that chromium will enhance the mutagenicity of sodium azide and 9-aminoacridine in the absence of significant toxicity. Neither action is dependent upon recA^+ regulated SOS repair pathways. Nickel and cadmium both increase mutagenicity following exposure to simple alkylating agents such as ethyl methanesulfonate. These responses cannot be explained by toxicity or by induction of error-prone repair. The interactions seem to be due to effects of metals on specific processes involved in the repair and/or replication of DNA following exposure to genotoxic agents.

Teratological studies are being conducted using Wistar Rats with various combinations of dimethoxyethyl phthalate (DMEP) and its metabolites and toluene, aspirin and caffeine. Interacting combinations of caffeine and aspirin or toluene and aspirin resulted in moderate potentiation of teratogenesis. Combinations of DMEP and caffeine, or DMEP and toluene and caffeine did not result in potentiation. An interesting finding is that DMEP and its metabolites cause a disorganization of cell position and tissue architecture in developing epithelial layers and this may be an inherent part of their mechanism of teratogenic action.

Biological interactions of two metals are being studied in rats and unequivocal results have been obtained in the production of an exaggerated nephrotoxic response when subthreshold doses of two metals were administered. Doses of mercuric chloride and potassium dichromate were administered subcutaneously. Renal slice transport of organic ions was monitored as an indication of the effects produced by the metals. Similar, although less dramatic effects are noted on overall renal function when the metals are given in combination as is true for tissue respiration. These experiments suggest the potential importance of exposure in the environment to even very low doses of certain substances.

In a study to determine factors which affect irritant potency of gases and aerosols, research efforts have been directed toward characterization of several aspects of ozone toxicity. A six-fold increase in the permeability of the respiratory mucosa of guinea pigs was evident 2 and 8 hours after ozone (1 ppm x 1 hour), but not at 24 hours. Histamine potentiated this response at 2 hours but not at 24 hours after ozone. The similar time course here to that

of ozone induced hypersensitivity to bronchoconstrictors suggests that alterations in mucosal function and perhaps in underlying structures (irritant receptors, mast cells) may contribute to airway hyperreactivity. Decreases in lung volumes and diffusing capacity up to 48 hours after ozone (1 ppm x 1 hour) have indicated that impaired pulmonary function occurs in animals with no outward signs of toxicity. Initial studies of prostaglandin levels suggest that these mediators are involved in producing these functional changes.

Studies are continuing on the enhancing effects of dietary zinc and of alcohol on methylbenzyl nitrosamine (MBN) carcinogenesis. Recent data suggest that zinc deficiency enhances MBN carcinogenesis by increasing levels of the promutagen O⁶-MEG and diminishing the capacity to remove it. Following MBN exposure, inhibition of ³H-thymidine was only temporary in the zinc deficient esophagus whereas the control esophagus required 10 days to return to pre-treatment levels. Hepatic DNA labeling was comparable in both control and in zinc-deficient rats. These findings indicate that increased cell turnover adduct formation, and decreased repair contribute to the increased susceptibility of the zinc-deficient esophagus to chemical carcinogenesis.

Changes in lung morphology, weight and epithelial cell kinetics in guinea pigs after exposure to ZnO+SO₂ aerosols are described in which changes occur in animals exposed for only 2-3 hours to the aerosols that contain both oxides in amounts well below current industrial exposure standards. The submicron zinc oxide particles are present at approximately 5 mg/m³ and are generated in the presence of 1 ppm sulfur dioxide and water vapor. Epithelia of the trachea and of the small airways (terminal bronchials, alveolar ducts) and also endothelial cells are injured.

Prior studies with rats have suggested that sulfur dioxide may potentiate the pulmonary carcinogenicity of polycyclic aromatic hydrocarbons, in particular benzo(a)pyrene (BP). Since man is also exposed simultaneously to these agents in urban and polluted atmospheres, he may be at risk from this combination. The effect of sulfite on the covalent binding of BP metabolites to cellular molecules in A549 cells was studied. Cellular protein was initially used as the nucleophilic trapping agent to react with electrophilic BP metabolites. Preliminary results indicated that when A549 cells were pre-exposed to sulfite (10 mM) for 2 hours followed by ³H-BP for an additional 4 hours, a 30 percent increase of covalently-bound radioactivity was observed.

One study has focused on the nephrotoxicity of S-(1,2-dichlorovinyl)-L-cysteine (DCVC) and related compounds. The role of pyridoxal phosphate-dependent enzymes in the nephrotoxicity of DCVC was proven by the synthesis and testing of S-(1,2-dichlorovinyl)-DL- α -methylcysteine, which cannot be metabolized by pyridoxal phosphate-dependent enzymes and blocked the nephrotoxicity of DCVC. To test the hypothesis that 1,2-dichlorovinyl mercaptan, which is a putative metabolite of DCVC is involved in the toxicity of DCVC, S-(1,2-dichlorovinyl)-L-homocysteine (DCVHC) was prepared. 1,2-Dichlorovinyl mercaptan should also be a metabolite of DCVHC. DCVHC was found to be extraordinarily nephrotoxic. Indeed, DCVHC may be the most potent organic nephrotoxin known. Finally, a role for γ -glutamyl transpeptidase in the nephrotoxicity of S-(1,2-dichlorovinyl)-glutathione (DCVG) was established; AT-125, a suicide substrate for γ -glutamyl transpeptidase, blocked the toxicity of DCVG.

In several acute toxicity studies, it has been shown that on a molar basis, selenium (Se) is the most effective cadmium antagonist known. The mechanism of this interaction in plasma and red blood cells has been shown to involve the metabolism of Se, as sodium selenite or selenide. The selenide then complexes with Cd^{2+} and is associated with proteins of specific molecular weights. The Cd in the Cd-Se-protein complex is believed to be biologically inactive. The present studies which employ low, chronic exposures to Cd by using diets differing in selenium and cadmium content are being conducted to establish the rat as a model for Cd-induced heart disease in humans.

Selenium compounds have been shown to antagonize the effects of mercury compounds in animal toxicity studies as well as in cytotoxicity studies in cell cultures. Studies are in progress to determine the possible effects of these compounds on the mercury-induced effects on nucleic acid synthesis in intact cells, isolated nuclei, and extracted polymerases. In addition, preliminary studies have shown that while methyl mercury (MeHg) is active in a cell transformation assay, the activity of MeHg together with N-methyl-N-nitro-N-nitrosoguanidine (MNNG) is considerably less than additive in this assay.

Long-term studies on the effects of ethanol on initiation and promotion of esophageal carcinogenesis are underway in rats. For the promotion studies, animals were initiated with methylbenzyl nitrosamine (MBN) for a period of time followed by treatment with isocaloric ethanol or control carbohydrate. Ethanol or control diets will be fed for a period of twelve weeks and the studies continued for a period of two years. Initiation studies will involve placing weanlings on an ethanol diet and subsequently exposed to MBN.

One study is concentrating on the enzymology of carbon tetrachloride metabolism and on the effect of hepatotoxic chemicals on liver calcium homeostasis. The carbon tetrachloride metabolism studies will seek to define the isozymes of cytochrome P-450 that are involved in the metabolism of carbon tetrachloride to phosgene and chloroform and the responsiveness of these isozymes to alcohol treatment. Carbon tetrachloride, chloroform, and bromobenzene administration leads to the appearance of glutathione S-transferase activity in blood and in an alteration of the chromatographic behavior of the transferases. Moreover, metabolites of bromobenzene and chloroform become covalently bound to the transferases. Thus, the transferases are released into blood from the liver after treatment with hepatotoxic chemicals.

Immunotoxicology

The role of environmental agents in producing adverse effects on the immune system is becoming more apparent with development and refinement of immunologic techniques applicable to such studies. Moreover, an appreciation for the role of xenobiotics in the induction of immunotoxicity has significantly increased. At present, however, only minimal information is available regarding the molecular events associated with chemical-induced immunotoxicity. For most xenobiotics, the ultimate product, molecular target and subsequent biochemical events responsible for derangements in the immune system are not well defined.

The NIEHS currently supports a variety of individual research projects in which immunology plays a key role.

Ten adolescent subjects with extrinsic asthma were studied during intermittent exercise exposure to filtered air, 0.5 ppm sulfur dioxide (SO_2) or $100 \mu\text{g}/\text{m}^3$ sulfuric acid (H_2SO_4). The purpose of this study was to compare changes in nasal power (the work of breathing) with pulmonary functional changes depending on the route of inhalation of the pollutants: oral inhalation through a mouthpiece or oronasal inhalation via a face mask. Statistically significant changes in total respiratory resistance, forced expiratory volume in one second (FEV_1) and maximal flow parameters ($\dot{V}_{\text{max}50}$ and $\dot{V}_{\text{max}75}$) were seen following exposures to SO_2 and H_2SO_4 . The magnitude of change in FEV_1 and $\dot{V}_{\text{max}50}$ was greater following oral compared to oronasal inhalation of SO_2 . This research demonstrates that SO_2 can cause nasal as well as pulmonary functional changes.

Cadmium is an environmental pollutant. The effects of cadmium on phagocytosis, a fundamental aspect of immunity, were studied in laboratory mice exposed to the metallic ion in drinking water for periods up to one year. There was a decrease in clearance from the circulation of ^{51}Cr labelled sheep red blood cells (E) and E coated with immunoglobulin G (IgG). This decrease was reversed when cadmium was removed from the drinking water. When organ uptakes of E and E-IgG were analyzed, it was found that as duration of cadmium exposure increased, the uptake in the liver decreased, whereas uptake in the spleen increased. Removal of the cadmium again reversed these uptake patterns towards normal despite a continuing high organ burden of cadmium, particularly in the liver. These studies are contributing to an understanding of the mechanism whereby cadmium affects phagocytosis.

Among the toxic effects which have been associated with the aromatic hydrocarbon (Ah) receptor in mice are hepatic enzyme induction, immunotoxicity, cleft-palate formation, and thymic atrophy. By using several PCB congeners and polycyclic aromatic hydrocarbons, evidence suggests that any ligand for this receptor should produce these effects. Correlations between Ah-receptor associated thymic atrophy and the suppression of antibody mediated immunity were studied. Using a T-independent antigen, it was demonstrated that this immunosuppression is not a consequence of thymic toxicity.

It has been established that chrysotile asbestos produces a 50 percent inhibition of migration of normal macrophages at about $100 \mu\text{g}/\text{ml}$. Tests also have established that the small standardized fibers are no more active than sonicated preparation of chrysotile. Furthermore, preparations of fibers exceeding 50 micrometers in length are more toxic than sonicated preparations of fibers less than 10 micrometers. The following observations have also been made: neither chrysotile, amosite or crocidolite can induce a burst in the hexose monophosphate shunt of BCG immune alveolar macrophages; chrysotile is the most toxic of the 3 forms tested as measured by migration inhibition and loss of viability of alveolar macrophages; complement is not involved in the toxicity of asbestos; and leaching of chrysotile with one normal HCl for 24 hours ($23-25^\circ\text{C}$) inactivates the toxicity for alveolar macrophages.

The host wide system of macrophages (the mononuclear phagocyte system or MPS) is a major target of many xenobiotics and environmental pollutants. Alterations in the MPS can be extremely damaging to the body in two distinct ways: depressed function of the MPS can lead to impaired resistance to the development of infections and tumors, while over-exuberance stimulation of the MPS

can lead to profound tissue injury damage and even carcinogenesis. An hypothesis was formulated that xenobiotics and environmental pollutants alter the development of the MPS, which is a dynamic system of cells whose state of development and activation is consistently in flux. A study of 7 environmental pollutants showed that 5 perturbed the development of the MPS. Some suppressed development, some stimulated development and some actually had both effects (that is, they induced early development and suppressed late development). One of the major changes induced in the MPS by xenobiotics is acquisition of competence to release reactive oxygen intermediates (ROI) such as hydrogen peroxide. The data strongly suggests the possibility that xenobiotics may perturb the mononuclear phagocyte system to secrete increased or aberrant amounts of ROI, which in turn can induce promutagenic changes in bystander normal cells.

Studies were carried out to evaluate both the autoimmune effects of different dosages of mercuric chloride and the kinetics of autoimmune responses to the glomerular basement membrane (GBM) observed in BN rats after the administration of mercury. Preliminary findings confirmed that very low amounts of mercuric chloride (10 micrograms/100g body weight) are still capable of inducing autoimmune responses to autoantigens of the GBM in 100 percent of animals. S.C. injection of mercuric chloride in similar low dosages has the same autoimmune effects in another inbred strain of rats (MAXX) but no effect in a third strain (M520). In addition, autoantibodies to GBM are present in the circulation by the ninth day after the first injection with mercuric chloride. Titers of autoantibodies to GBM reach a peak by day 12-15 and then decrease rapidly, reaching baseline values in the following 2 weeks. These results have been obtained by ELISA and PFC assays. These findings suggest that mercury compounds may be an important cause of autoimmune disease resulting from environmental pollution.

Asbestos-related diseases are a major public health problem in the U.S. An estimated 14 million individuals have been exposed to asbestos during their working lives. Asbestos has been casually linked to interstitial lung disease, pleural fibrosis, lung cancer, mesothelioma and cancers of the gastrointestinal tract, larynx and possibly, kidney. Altered immunoregulation most likely plays a role in the pathogenesis of asbestos-related diseases. Preliminary results have suggested increases in T-lymphocyte subsets OKT3, OKT8 and OKT14 in bronchoalveolar lavage (BAL) and in OKIa in peripheral blood and BAL from patients with asbestos exposure. In addition, differences in expression of antigen by peripheral blood monocytes and alveolar macrophages have been found.

Studies are underway to examine the effects of cadmium, lead, benzene, and 7,12 dimethylbenz(a)anthracene (DMBA) on the immune system of young adult female B6C3F1. Previous studies have shown that a dose of 12 mg/kg lead acetate or 0.9 mg/kg cadmium acetate given i.p. depresses the humoral immune response 3 days later to sheep erythrocytes (SRBC) and TNP-Ficoll, while not affecting the primary immune response to TNP-LPS. No significant alteration in cell surface markers (Mac-1, Lyt-1, Lyt-2, Thy 1.2, and pre-B/B cell antigen 14.8) was detected in the spleens of the cadmium or lead-treated animals examined on days 1, 3, and 5 after exposure. A significant increase in the number of large cells was noted in the bone marrow following an acute exposure

to cadmium and lead at the doses listed above. A significant decrease in the number of bone marrow cells expressing Mac-1, Lyt-1, 14.8, and a monocyte/lymphoid marker known as 55-7.2 was observed.

A number of industrial and environmental chemicals are known to cause allergic asthmatic reactions in exposed individuals. An animal model is being developed for this response in order to determine: (a) the potency of various chemicals for causing sensitization, (b) the mechanism underlying chemical sensitization, and (c) the permanence of sensitization. In the model, guinea pigs are exposed via the inhalation route to known concentrations of chemicals. Following a two-week rest period, re-exposure of animals to low concentrations of the chemicals has resulted in elicitation of respiratory sensitivity reactions. Using this procedure, sensitization has been achieved to toluene diisocyanate (TDI), a major component of polyurethane and to bacterial subtilisin, an enzyme used in laundry detergents. The sensitization was found to be concentration-dependent since exposure to high concentrations of chemicals resulted in a great percent of animals developing sensitivity and in a large number of severe sensitization responses. By comparison, exposure to low concentrations of these materials produced sensitization in very few animals. Recognition of this relationship and comparison of the concentration of chemicals required to achieve sensitization in 50 percent of the animals (SD_{50}) will enable comparison of the potencies of chemicals as respiratory tract sensitizers.

The effects of acute exposure to both lead and cadmium in adult mice have been evaluated. The data showed that both metals affect antibody formation to T-dependent and T-independent antigens differently and suggest that there is a differential susceptibility of T and B lymphocytes to these immunotoxic agents. Further in vitro studies in adults confirmed this finding and also showed a transient, enhancing effect at the level of the B cell. Acute exposure of newborn mice to lead appears to produce an effect on T-dependent antibody formation of somewhat longer duration than that observed in adults; but, with time, this depressive effect was no longer evident. An enhancing effect on T-independent responses, presumably manifested at the level of the B cell, was of longer duration. The implications of the latter finding on host immune resistance are not known.

Several studies conducted during the past year have focused on understanding the cell biology of macrophage ($M\phi$) differentiation and alterations induced by exposure to N-nitrosodimethylamine (DMN). Previous results had demonstrated that daily exposure to the carcinogen DMN affected cell-mediated immunity through changes in $M\phi$ function. The effects of DMN on the differentiation of $M\phi$ from marrows of treated animals were examined. DMN produced minimal change in the number of CFU-GM after 7d of culture. The adherent $M\phi$ population was characterized by lower numbers of cells in S-phase and a decrease in the number of cells expressing Ia antigens. Bone marrow derived $M\phi$ (BMDM) from vehicle control animals reconstituted the KLH proliferative response to T cells from both vehicle and DMN exposed animals further demonstrating that T cells from DMN treated animals are responsive; whereas, $M\phi$ from such animals have altered functional capabilities. These studies suggest that DMN treatment results in a decrease in the number of $M\phi$ expressing Ia antigen

while these cells have increased effector cell function (tumor cell killing). DMN exposed animals also exhibited a significant reduction in the number of pulmonary tumor nodules and a 10-fold increase in cytostasis activity in vitro when challenged with the B16 melanoma.

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